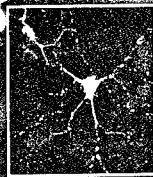


INTERNATIONAL SYMPOSIUM



**The Cholinergic Synapse:
Structure, Function and
Regulation**

November 6-10, 1994

Julius Axelrod
Honorary Chairman

19950303 000

University of Maryland School of Medicine

*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

Invited Speakers

<i>Albuquerque, E.X.</i>	<i>Massoulié, J.</i>
<i>Arneric, S.</i>	<i>McMahan, U.J.</i>
<i>Barde, Y.-A.</i>	<i>Merlie, J.P.</i>
<i>Barnard, E.A.</i>	<i>Nakanishi, S.</i>
<i>Betz, H.</i>	<i>Oppenheim, R.</i>
<i>Blaustein, M.P.</i>	<i>Patrick, J.</i>
<i>Bloch, R.J.</i>	<i>Poo, M.-m.</i>
<i>Carr, J.</i>	<i>Rahamimoff, R.</i>
<i>Changeux, J.-P.</i>	<i>Randall, W.</i>
<i>Cohen, J.B.</i>	<i>Role, L.W.</i>
<i>Colquhoun, D.</i>	<i>Rosenberry, T.</i>
<i>Conti-Fine, B.M.</i>	<i>Rotundo, R.L.</i>
<i>Cull-Candy, S.</i>	<i>Sakmann, B.</i>
<i>Dani, J.</i>	<i>Salpeter, M.</i>
<i>Doctor, B.P.</i>	<i>Scheller, R.H.</i>
<i>Dolly, J.O.</i>	<i>Seeburg, P.</i>
<i>Fischbach, G.D.</i>	<i>Shafferman, A.</i>
<i>Froehner, S.</i>	<i>Silinsky, E.M.</i>
<i>Fuchs, S.</i>	<i>Silman, I.</i>
<i>Greengard, P.</i>	<i>Soreq, H.</i>
<i>Hall, Z.</i>	<i>Südhof, T.C.</i>
<i>Heinemann, S.</i>	<i>Sussman, J.</i>
<i>Hucho, F.</i>	<i>Taylor, P.</i>
<i>Huganir, R.</i>	<i>Tsien, R.</i>
<i>Jahn, R.</i>	<i>Tzartos, S.J.</i>
<i>Karlin, A.</i>	<i>Unwin, N.</i>
<i>Llinás, R.</i>	<i>Westbrook, G.</i>
<i>Lindstrom, J.</i>	<i>Wonnacott, S.</i>
<i>Maelicke, A.</i>	

Invited Chair/Co-Chair

Alger, B.
Aracava, Y.
Burt, D.
Daly, J.
Drachman, D.
Eldefrawi, A.
Eldefrawi, M.
Fambrough, D.
Frost, D.O.
Inesi, G.
Krueger, B.
Pumplin, D.
Shipley, M.T.
Van der Kloot, W.
Weinreich, D.

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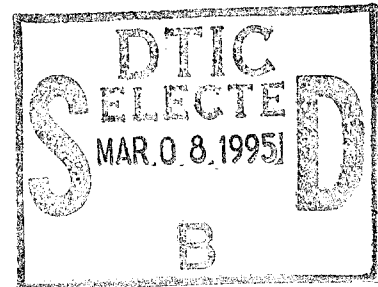
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AND REGULATION (CONFERENCE - NOVEMBER 6-10, 1994)

PRINCIPAL INVESTIGATOR: Edson X. Albuquerque, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Maryland
School of Medicine
Department of Pharmacology
655 West Baltimore Street
Baltimore, Maryland 21201-1559

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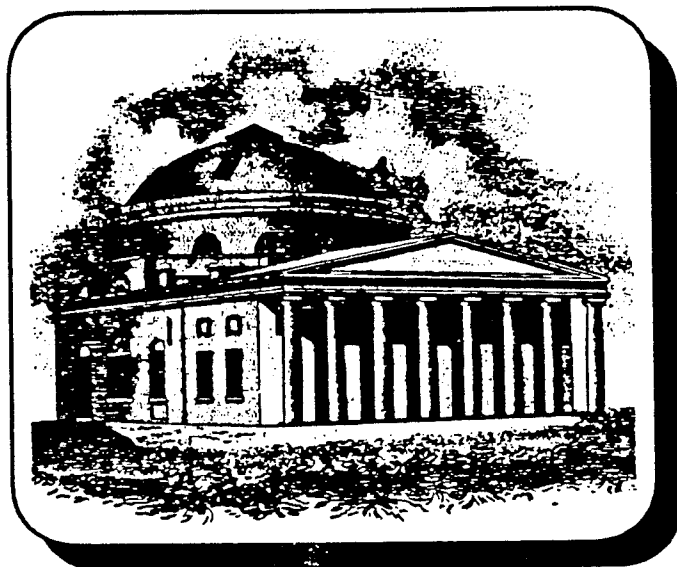
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**THE CHOLINERGIC SYNAPSE:
STRUCTURE, FUNCTION AND REGULATION.**



*Historic Davidge Hall, University of Maryland at Baltimore,
School of Medicine*

*Third International Symposium on The Cholinergic Synapse
University of Maryland School of Medicine
Baltimore, Maryland, November 6-10, 1994*

Honorary chairman: Dr. Julius Axelrod

Program and Abstracts

Organizers:

E.X. Albuquerque

F. Hucho

R. Rahamimoff

I. Silman

*Local organizers: E.X. Albuquerque, M.P. Blaustein, R. Bloch, D. Frost,
R. Huganir, W. Randall, and D. Weinreich.*

Note: The abstracts in this volume have been neither reviewed nor edited.

GENERAL INFORMATION

LOCATION

Stouffer Harborplace Hotel, Baltimore's Inner Harbor, Baltimore, Maryland.
The speaker's presentations will take place in the Maryland Ballroom, and the Krantz Lecture will be given at the Maryland Science Center.

REGISTRATION DESK

The registration desk will be open on Sunday, November 6th, from 16:00 to 18:00 and mornings during the meeting. The telephone number is (410)547-1200 ext 3002.

BADGES

Members of the local organizing committee are identified by a blue ribbon, and the staff are identified by a white ribbon. They will be pleased to assist you.

SLIDES

Speakers are invited to preview and load their slides at the speaker-ready room. Lectures are planned for twenty minutes plus ten minutes for discussion.

POSTERS

All poster presentations will take place in the salons adjacent to the Maryland Ballroom at the Stouffer Harborplace Hotel. On November 8th, all poster presenters are invited to put up their posters on the board bearing their poster number. This number can be found in this program book. The posters are classified into eight sessions according to their main topic. Presenters of posters included in sessions 1 and 2 should be at their posters on Tuesday from 10:00 to 10:30. Presenters of posters included in sessions 3 and 4 should be at their posters on Tuesday from 15:00 to 15:30. Presenters of posters included in sessions 5 and 6 should be at their posters on Wednesday from 10:30 to 11:30. Presenters of posters included in session 7 should be at their posters on Wednesday from 15:30 to 17:30. Presenters of posters included in session 8 should be at their posters on Thursday from 10:20 to 10:50. All posters should be removed at the end of the Symposium.

ADDENDUM

Please note that there is a change in one of the scheduled progress reports. On Thursday, November 10, the 9:20 a.m. speaker is G.K. Lloyd; his topic is "Subtype-selective cholinergic ion channel agonists as potential antiparkinson agents." The abstract appears as the last page of this book, following the index.

Please excuse an error in the heading for the first Thursday morning session of progress reports. The date should read *Thursday, November 10*.

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THE SPECIAL LECTURES

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The John C. Krantz Lecture by Dr. Bert Sakmann

SYMPOSIUM PRESENTATIONS

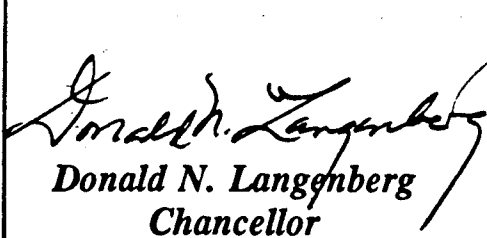
- Session 1: Synaptic Modulation*
- Session 2: Synaptic Modulation and Phosphorylation*
- Session 3: Nicotinic Acetylcholine Receptor*
- Session 4: Synaptogenesis and Development I*
- Session 5: Synaptogenesis and Development II*
- Session 6: CNS Receptor Function and Structure I*
- Session 7: CNS Receptor Function and Structure II*
- Session 8: Progress Reports by Selected Speakers*
- Session 9: Cholinesterase I*
- Session 10: Cholinesterase II*


POSTER PRESENTATIONS

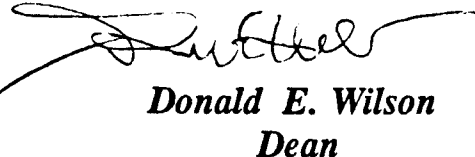
- Session 1: Receptor Assembly and Structure*
- Session 2: Synaptogenesis and Developmental Neurobiology*
- Session 3: Receptor Regulation and Modulation*
- Session 4: Receptor Gene Expression*
- Session 5: Cholinesterases*
- Session 6: Neurotoxins*
- Session 7: Presynaptic Mechanisms*
- Session 8: Pharmacology of Nicotinic Receptors*

WELCOME

The University of Maryland School of Medicine is pleased to host the Third International Symposium on Cholinergic Synapses. The University would like to welcome all of you and to acknowledge the major contributions that you have made to the understanding of brain function. This is the first time that such an outstanding group of scientists has gathered at our Institution to discuss the molecular basis of the integrative functions of the central nervous system. The outcome of this meeting will be critical to the "Decade of the Brain", and our University is extremely proud to be a part of such an event. Welcome to Baltimore.


Donald N. Langenberg
Chancellor


David J. Ramsay
President


Donald E. Wilson
Dean

PROGRAM

SUNDAY, NOVEMBER 6, 1994

16:00-18:00 REGISTRATION

MONDAY, NOVEMBER 7, 1994

8:00 Welcome, Drs. Donald E. Wilson (Dean, University of Maryland School of Medicine) and E.X. Albuquerque (Chairman of the Symposium on Cholinergic Synapse).

OPENING REMARKS, DR. JULIUS AXELROD (Honorary Chairman)

SYNAPTIC MODULATION

Chair: Dr. S. Nakanishi; Co-Chair: Dr. D. Weinreich

- 8:30 Scheller, R.H. Molecular Mechanisms of Synaptic Transmission
9:00 Blaustein, M.P. Regulation of Cytosolic Free Ca^{2+} and Endoplasmic Reticulum Ca^{2+} stores in Neurons and Astrocytes: Role of the Na/Ca Exchanger
9:30 Rahamimoff, R. An Analysis of the Function of the Cholinergic Nerve Terminal: Ion Channels, Confocal Calcium Images and Oscillations.
10:00 -- Coffee Break

Chair: Dr. D. Fambrough; Co-Chair: Dr. M.T. Shipley

- 10:30 Jahn, R. Molecular Mechanisms of Synaptic Vesicle Docking and Fusion
11:00 Llinás, R. The Role of Calcium Concentration Microdomains in Neuronal Integration and Synaptic Transmission
11:30 Dolly, J.O. Neuronal Voltage-Sensitive K^+ Channels: Structural and Functional Properties
12:00 Südhof, T.C. Molecular Mechanisms Regulating Neurotransmitter Release
12:30 -- Lunch Break

SYNAPTIC MODULATION AND PHOSPHORYLATION

Chair: Dr. P. Greengard; Co-Chair: Dr. G. Inesi

- 13:30 Westbrook, G. Regulation of Glutamate Channels at Central Synapses
14:00 Tsien, R. Ca^{2+} Channels in CNS Neurons and Neurotransmission at the Level of Single Synaptic Boutons
14:30 Dani, J. Calcium Modulation and Calcium Permeability of Nicotinic Acetylcholine Receptors
15:00 -- Coffee Break

Chair: Dr. S. Heinemann; Co-Chair: Dr. Y. Aracava

- 15:30 Silinsky, E.M. What is the Cause of Neuromuscular Depression at Physiological Levels of Acetylcholine Release?
- 16:00 Haganir, R. Regulation of Neurotransmitter Receptors by Protein Phosphorylation
- 16:30 Role, L.W. Long-Term Regulation of Nicotinic Acetylcholine Receptor Expression and Ion Channel Function in Neurons
- 17:00 Barnard, E.A. The Receptor Families of Ion Channels Gated by Extracellular Transmitters, from Nematode to Man
- 17:30 -- Refreshments
- 18:15-19:45 Grollman Memorial Lecture -- Prof. P. Greengard
Pre- and Post-synaptic Modulation of Synaptic Transmission

TUESDAY, NOVEMBER 8, 1994

NICOTINIC ACETYLCHOLINE RECEPTOR

Chair: Dr. D. Drachman; Co-Chair: Dr. D. Pumplin

- 8:00 Cohen, J.B. Membrane Spanning Regions of the Nicotinic Acetylcholine Receptor: Structure of the Protein-Lipid Interface and the Ion Channel
- 8:30 Karlin, A. Structures Involved in Binding, Gating, and Conduction in Acetylcholine Receptors
- 9:00 Hucho, F. Investigations of the Secondary Structure of the Nicotinic Acetylcholine Receptor
- 9:30 Unwin, N. Three-Dimensional Structure of the Acetylcholine Receptor in the Closed and Open States
- 10:00 -- Coffee Break -- Poster Session

Chair: Dr. J. Daly; Co-Chair: Dr. M. Eldefrawi

- 10:30 Hall, Z. Agrin and the Assembly of the Neuromuscular Junction
- 11:00 Heinemann, S. Alpha 9: A New Acetylcholine Receptor with Novel Pharmacological Properties
- 11:30 Changeux, J.-P. Functional Organisation and Dynamics of the Nicotinic Receptors: Recent Developments
- 12:00 -- Lunch Break

SYNAPTOGENESIS AND DEVELOPMENT I

Chair: Dr. Z. Hall; Co-Chair: Dr. D. Burt

- 13:00 McMahan, U.J. Composition and Function of the Matrix in the Synaptic Cleft
- 13:30 Salpeter, M. Building a Stable Neuromuscular Junction
- 14:00 Fischbach, G.D. A Role for Tyrosine Kinases in Synapse Formation
- 14:30 Merlie, J.P. The Role of 43K Rapsyn in Synapse Formation and Stabilization
- 15:00 -- Coffee Break -- Poster Session

SYNAPTOGENESIS AND DEVELOPMENT II

Chair: Dr. A. Karlin; Co-Chair: Dr. D.O. Frost

- 15:30 Poo, M.-m. Activity-Dependent Modulation of Developing Neuromuscular Synapses
- 16:00 Bloch, R.J. A Spectrin-Based Membrane-Skeleton Involved in AChR Clustering
- 16:30 Oppenheim, R. Activity-Dependent Differentiation and Survival of Developing Signal Motoneurons
- 17:00 Barde, Y.-A. Regulation of BDNF mRNA Levels During the Development of Chick Visual System
- 17:30 -- Group to walk to the Maryland Science Center
- 18:00 -- Refreshments (*at the Maryland Science Center*)
- 18:45-20:15 **Krantz Lecture--Prof. B. Sakmann**
Molecular Basis and Functional Consequences of the γ/ϵ -AChR Subunit Switch at the Rat Neuromuscular Junction (*at the Maryland Science Center*)

WEDNESDAY, November 9, 1994

CNS RECEPTOR FUNCTION AND STRUCTURE I

Chair: Dr. S. Fuchs; Co-Chair: Dr. B. Alger

- 8:00 Patrick, J. Cyclophilin-Dependent Expression of $\alpha 7$ Neuronal Nicotinic Acetylcholine Receptor
- 8:30 Lindstrom, J. Neuronal Nicotinic $\alpha 3$, $\alpha 4$, $\alpha 7$, and $\alpha 8$ AChRs
- 9:00 Albuquerque, E.X. Functional and Structural Aspects of Neuronal Nicotinic Receptors
- 9:30 Maelicke, A. Noncompetitive Agonism at Nicotinic Acetylcholine Receptors
- 10:00 Colquhoun, D. Comparison of Native and Recombinant Receptor Channels: Glutamate and Acetylcholine Receptors
- 10:30 -- Coffee Break -- Poster Session

CNS RECEPTOR FUNCTION AND STRUCTURE II

Chair: Dr. J.-P. Changeux; Co-Chair: Dr. B. Krueger

- 11:30 Cull-Candy, S. Glutamate-Channels and Synaptic Transmission in Cerebellar Granular Cells
- 12:00 Nakanishi, S. Molecular Diversity and Physiological Functions of Glutamate Receptors
- 12:30 Seeburg, P. Glutamate-Gated Ion Channels in Brain: Properties and Genetic Control
- 13:00 Betz, H. The Inhibitory Glycine Receptor: Structure, Mutants, and Postsynaptic Localization
- 13:30 -- Afternoon free
- 15:30 -- Poster Session

THURSDAY, NOVEMBER 10, 1994

PROGRESS REPORTS BY SELECTED SPEAKERS

Chair: Dr. T. Narahashi; Co-Chair: Dr. W. Van der Kloot

- 8:00 Froehner, S. The Submembrane Machinery for Acetylcholine Receptor Clustering
- 8:20 Tzartos, S.J. Acetylcholine Receptor Tyrosine and Serine Phosphorylation, Monoclonal Antibodies as Site-Specific Tools for Phosphorylation and Channel Function
- 8:40 Fuchs, S. Mapping of Functional Sites at the Nicotinic Acetylcholine Receptor
- 9:00 Conti-Fine, B.M. A "Neuronal" Nicotinic Acetylcholine Receptor Regulating Cell Adhesion is Expressed in Human Epidermal Keratinocytes
- 9:20 Wonnacott, S. Presynaptic Nicotinic Receptor Modulation of Dopamine Release in Rat Brain
- 9:40 Americ, S. Cholinergic Channel Activators (ChCAs) for the Potential Treatment of Alzheimer's Disease
- 10:00 Doctor, B.P. Modulation of Catalysis and Inhibition of Acetylcholinesterase by Monoclonal Antibodies
- 10:20 -- Coffee Break -- Poster Session

CHOLINESTERASE I

Chair: Dr. B. Sakmann; Co-Chair: Dr. R. Bulleit

- 10:50 Silman, I. Studies of Partially Unfolded States of *Torpedo californica* Acetylcholinesterase
- 11:10 Shafferman, A. Molecular Aspects of Catalysis and of Allosteric Regulation of Acetylcholinesterase
- 11:30 Taylor, P. Expression and Ligand Specificity of Acetylcholinesterase and the Nicotinic Receptor: A Tale of Two Cholinergic Sites
- 12:00 Massoulié, J. Molecular Forms of Acetylcholinesterase: Structure and Interactions
- 12:30 Sussman, J. 3-D Structure of Acetylcholinesterase and Complexes of it with Anticholinesterase Agents
- 13:00 -- Lunch Break

CHOLINESTERASE II

Chair: Dr. B.P. Doctor; Co-Chair: Dr. A. Eldefrawi

- 14:30 Rotundo, R.L. Compartmentalized Regulation of Acetylcholinesterase Expression in Skeletal Muscle
- 15:00 Randall, W.R. Neural Regulation of Acetylcholinesterase Gene Expression
- 15:30 Soreq, H. Engineering Cholinergic Synapses Through Overexpression of Human Cholinesterase in *Xenopus* Tadpoles and Transgenic Mice
- 16:00 Rosenberry, T. Genetic Analysis of Glycoinositol Phospholipid (GPI) Anchor Function in *Drosophila* Acetylcholinesterase
- 16:30 Final remarks E. X. Albuquerque, I. Silman, R. Rahamimoff, F. Hucho.

NOTES

THE SPECIAL LECTURES

THE AARON I. GROLLMAN MEMORIAL LECTURE

Lecturer: Dr. Paul Greengard

The Faculty of the Department of Pharmacology and Experimental Therapeutics of the University of Maryland School of Medicine extends to you a cordial invitation to

THE AARON I. GROLLMAN MEMORIAL LECTURE

The Dr. Aaron I. Grollman Visiting Professorship was established and endowed in 1981 by Ellis Grollman, School of Pharmacy, class of 1926, in memory of his brother Aaron, School of Medicine, class of 1928. Dr. Aaron I. Grollman was chief of surgery at the Cincinnati Jewish Hospital. Dr. Ellis Grollman practiced at the Johns Hopkins University Hospital and in Ocean City, Gaithersburg and Annapolis, MD. Income from the endowment brings internationally recognized visiting professors to the University of Maryland School of Medicine to deliver the Dr. Aaron I. Grollman Memorial Lecture. The award is rotated annually among the medical school's basic science departments (Anatomy, Biological Chemistry, Biophysics, Microbiology & Immunology, Pharmacology & Experimental Therapeutics, and Physiology).

Former Grollman Professors and Presenting Departments

- | | |
|---------------------------------|---|
| <i>Dr. Arthur Kornberg</i> | <i>- Pharmacology & Experimental Therapeutics, 1982</i> |
| <i>Dr. D. Carleton Gajdusek</i> | <i>- Microbiology & Immunology, 1983</i> |
| <i>Dr. Han Bogind Khorana</i> | <i>- Biological Chemistry, 1984</i> |
| <i>Dr. Torsten N. Wiesel</i> | <i>- Physiology, 1985</i> |
| <i>Dr. Elizabeth Hay</i> | <i>- Anatomy & Cellular Biology, 1987</i> |
| <i>Dr. Bertil Hille</i> | <i>- Biophysics, 1988</i> |
| <i>Dr. Robert J. Lefkowitz</i> | <i>- Pharmacology & Experimental Therapeutics, 1989</i> |
| <i>Dr. Ernesto Carafoli</i> | <i>- Biological Chemistry, 1994</i> |

1994

Prof. Paul Greengard
Molecular and Cellular Neuroscience
Rockefeller University

THE JOHN C. KRANTZ, JR. LECTURE

Lecturer: Dr. Bert Sakmann

The Faculty of the Department of Pharmacology and Experimental Therapeutics of the University of Maryland School of Medicine extends to you a cordial invitation to

THE JOHN C. KRANTZ, JR. LECTURE IN PHARMACOLOGY

The John Christian Krantz, Jr. Lectureship was established in June, 1966, by friends and colleagues of Dr. Krantz upon the occasion of his retirement from the faculty of the School of Medicine. The purpose of the John C. Krantz, JR. Lectureship in Pharmacology and Experimental Therapeutics is to provide lectures at the University of Maryland School of Medicine in the general areas of Molecular Pharmacology, Cell Biology, and Molecular Biology. This lecture is the twenty-fifth in the series given by outstanding scientists from the United States and abroad.

Former Lecturers

Oliver H. Lowry, M.D. Ph.D.	1969	Motoy Kuno, M.D.	1979
Harry Eagle, M.D.	1970	Eric R. Kandel, M.D.	1980
Linus Pauling, Ph.D.	1972	Sir Andrew Huxley	1982
Julius Axelrod, Ph.D.	1975	Pedro Cuatrecasas, M.D.	1982
George B. Koelle, M.D. Ph.D.	1975	Albert J. Aguayo, M.D.	1983
Edward Reich, M.D. Ph.D.	1975	Bernhard Witkop, Ph.D.	1985
Edward A. Kravitz, Ph.D.	1975	John Daly, Ph.D.	1985
Marshall W. Nirenberg, Ph.D.	1976	Arthur Karlin, Ph.D.	1985
Rodolfo R. Llinas, M.D. Ph.D.	1976	David Colquhoun, Ph.D.	1987
Elliot S. Vesell, M.D.	1977	Palmer Taylor, Ph.D.	1988
Daniel C. Tosteson, M.D.	1977	Dale Purves, M.D.	1991
J.-P. Changeux, Ph.D.	1978	Toshio Narahashi, Ph.D.	1992
Sir Bernard Katz	1979		

1994

Prof. Bert Sakmann
Abteilung Zellphysiologie
Max-Planck-Institut für Medizinische Forschung
Heidelberg, Germany

NOTES

**ABSTRACTS
SYMPOSIUM PRESENTATIONS**

MONDAY, NOVEMBER 7, 1994

(MORNING, FIRST SESSION)

SYNAPTIC MODULATION

Chair: Dr. S. Nakanishi; Co-Chair: Dr. D. Weinreich

- | | | |
|-------|-----------------|--|
| 8:30 | Scheller, R.H. | Molecular Mechanisms of Synaptic Transmission |
| 9:00 | Blaustein, M.P. | Regulation of Cytosolic Free Ca^{2+} and Endoplasmic Reticulum Ca^{2+} stores in Neurons and Astrocytes: Role of the Na/Ca Exchanger |
| 9:30 | Rahamimoff, R. | An Analysis of the Function of the Cholinergic Nerve Terminal: Ion Channels, Confocal Calcium Image and Oscillations |
| 10:00 | -- | Coffee Break |

MOLECULAR MECHANISMS OF SYNAPTIC TRANSMISSION. Scheller, R.H.
Howard Hughes Medical Institute Research Laboratories, Stanford University Medical
Center, Stanford CA 94305

A description of the molecular mechanisms that underlie neurotransmitter release constitutes one of the major goals in cellular and molecular neurobiology. Such a description will provide insight into normal synaptic function and its physiological and pathological modification. Two of the central steps in the process of neurotransmitter release are the interaction, or docking, of synaptic vesicles with the presynaptic plasma membrane and the calcium-triggered fusion of these two membranes in response to stimulation. As synaptic vesicles play a central role in both the storage and exocytosis of neurotransmitters, they have been extensively studied at a molecular level. These studies have led to the identification and characterization of proteins, both in the synaptic vesicle membrane and in the presynaptic plasma membrane, that are likely to participate in synaptic vesicle docking and fusion.

Studies on synaptic vesicle docking and fusion have recently converged with those on the mechanisms responsible for constitutive membrane trafficking. The identification of three synaptic membrane proteins that are components of a receptor for the general membrane trafficking factors NSF (*N*-ethylmaleimide-sensitive factor) and SNAPs (soluble NSF attachment proteins) is a striking example of this convergence. NSF and SNAPs, initially identified as soluble factors required for constitutive vesicle-mediated transport within the Golgi complex, participate in vesicle targeting and fusion at multiple stages of the secretory and endocytic pathways. A complex that includes NSF, SNAPs and solubilized membrane proteins is recovered at 20S on glycerol gradients. The stability of this 20S complex is controlled by the hydrolysis of ATP, presumably by the ATPase activity associated with NSF. Söllner *et al.* took advantage of this regulation by ATP to identify the membrane components of the 20S complex from brain as three synaptic proteins: the presynaptic plasma membrane proteins syntaxin, SNAP-25, and the synaptic vesicle protein VAMP (also known as synaptobrevin).

The significance of the 20S complex in synaptic transmission is emphasized by the observation that each of its membrane components is a potential target for the inhibitory action of clostridial neurotoxins. Clostridial neurotoxins, which include tetanus and botulinum toxins, are efficient inhibitors of neurotransmitter release. Insight into the potential mechanism of action of these toxins has been provided by the demonstration that the light chains of tetanus toxin and botulinum-B toxin are zinc endoproteases that selectively cleave VAMP. Further studies have demonstrated that SNAP-25 and syntaxin are substrates for endoprotease

respectively. Although the cleavage of VAMP, syntaxin, and SNAP-25 provide an attractive model for clostridial neurotoxin action, other protease targets or mechanisms of action may also exist.

The general involvement of the 20S complex in membrane trafficking is suggested by the fact that each of its components has homologs that are required for the proper functioning of the secretory pathway in the yeast *Saccharomyces cerevisiae*. In addition, resealing of the plasma membrane in response to injury is inhibited by botulinum neurotoxins A and B, which implies that isoforms of two of the 20S complex components, VAMP and SNAP-25, play a role in the more general membrane trafficking events that underlie this process.

Biochemical characterization of the assembly and disassembly of the 20S complex, and the identification of a 7S intermediate complex, have provided further insight into the regulation of synaptic vesicle docking and fusion. In the absence of NSF and SNAPs, the three membrane components of the 20S complex (VAMP, syntaxin, and SNAP-25) are recovered along with synaptotagmin in a complex that sediments at 7S following detergent solubilization. Addition of α -SNAP results in the displacement of synaptotagmin from the 7S complex, whereas subsequent addition of NSF produces the 20S complex. The hydrolysis of ATP by NSF results in the near complete disruption of the 20S complex so that even the interactions that defined the 7S complex are no longer detected.

An additional component that may participate in the regulation of synaptic vesicle docking and fusion is a mammalian homolog of the *C. elegans unc-18* and yeast *secl* gene products, termed munc-18 and n-secl, respectively. Although n-secl interacts directly with syntaxin, consistent with the genetic interaction between *secl* and the yeast syntaxin genes SSO1 and SSO2, it is not readily detected as a component of either the 7S or 20S protein complexes. This suggests that n-secl may exert its effect either before the formation of the 7S complex or after the disassembly of the 20S complex. The former possibility is supported by the accumulation of post-Golgi transport vesicles in *secl* mutant yeast.

The emerging similarities between constitutive and regulated membrane trafficking discussed above suggest that the molecular mechanisms that mediate synaptic vesicle docking and fusion are closely related to those that mediate transport vesicle docking and fusion at other stages of the secretory and endocytic pathways. If this is the case, the regulatory events that control synaptic vesicle docking and fusion may simply consist of the removal of a block or clamp on the constitutive fusion machinery. The apparent competition between synaptotagmin, implicated as a potential negative regulator from genetic studies, and α -SNAP in the formation of the 20S complex may be such a regulatory event.

The specificity of vesicle docking and fusion requires a regulatory event essential to all membrane trafficking pathways. The rab family of low molecular weight GTP-binding proteins has been implicated in vesicle targeting in both the secretory and endocytic pathways. Recent studies of two yeast rab proteins, sec4p and ypt1p, indicate, however, that they are not the sole determinants of membrane trafficking specificity. Like the rab proteins, yeast Sec1p and the yeast homologs of VAMP (Snc1p and Snc2p and syntaxin (Sso1p and Sso2p) are each members of small protein families where a different member is required at distinct stages of the secretory pathway. This suggests that these proteins may also contribute to the specificity of transport vesicle targeting and fusion. The identification of broadly expressed mammalian isoforms of VAMP and syntaxin and the demonstration of direct interactions between some, but not all, syntaxin isoforms and both VAMP and n-secl further supports this possibility. These observations indicate that the specificity inherent in vesicle-mediated membrane transport may be mediated by a combination of reactions involving at least four families of proteins (syntaxin, VAMP, sec1 and rab). That three of these protein families (syntaxin, VAMP and sec1) may participate in the assembly of the 7S complex emphasizes the potential importance of this complex in determining the specificity of vesicle targeting.

REGULATION OF CYTOSOLIC FREE Ca^{2+} AND ENDOPLASMIC RETICULUM Ca^{2+} STORES IN NEURONS AND ASTROCYTES: ROLE OF THE Na/Ca EXCHANGER

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Ca^{2+} ions are involved in signalling in virtually all cells of higher animals. In the brain, Ca^{2+} signalling is of paramount importance in synaptic transmission because a rise in the cytosolic free Ca^{2+} concentration, $[\text{Ca}^{2+}]_{\text{cyt}}$, triggers neurotransmitter release. Cytosolic Ca^{2+} is also involved in nerve growth cone motility, and in the regulation of many key enzymes including some protein kinases (C-kinases) and phosphatases. Numerous recent studies also indicate that cytosolic Ca^{2+} is involved in signalling in astrocytes. Indeed, complex intercellular signalling patterns, involving elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ in both astrocytes and neurons, may represent a widespread non-synaptic mechanism of communication in the nervous system. Moreover, defective Ca^{2+} regulation and Ca^{2+} overload in neurons and astrocytes is associated with cell injury and cell death.

The two main sources of Ca^{2+} for cell signalling are the extracellular fluid (ECF) and the intracellular stores. There are multiple intracellular stores of Ca^{2+} , with different properties; however, the endoplasmic reticulum (ER, which appears to be heterogeneous) is the main source of intracellular "second messenger" Ca^{2+} (Pozzan et al., 1994).

At presynaptic nerve terminals, much of the signal Ca^{2+} comes from the ECF, and enters the cells via voltage-gated Ca^{2+} channels in the plasmalemma. In other parts of the neurons and, especially in astroglial cells, which occupy a large fraction of the brain and closely invest most synapses, the ER may be the dominant source of signal Ca^{2+} . This ER Ca^{2+} is released by inositol trisphosphate (IP_3), by Ca-induced-Ca release, and perhaps by cyclic ADP-ribose (Pozzan et al., 1994).

Whether the signal Ca^{2+} is derived from the ER or from the ECF, the ER may play a central role in Ca^{2+} -dependent signalling. The ER may serve either as the main storage site for releasable Ca^{2+} or, where Ca^{2+} influx is important, as a major buffer site until the entering Ca^{2+} can be extruded during recovery from excitation to restore Ca^{2+} homeostasis. Often overlooked, however, is the possibility that the ER stores may be heterogeneous, and that they may be modulated by, for example, cell activity. This is central to cell function because, if the amount of Ca^{2+} released is directly related to the fractional saturation of the store, the regulation of releasable Ca^{2+} in the stores may profoundly influence the responsiveness of cells to activating influences such as depolarization, neurotransmitters, and various other agonists.

We have suggested that the plasmalemmal Na^+ pump and Na/Ca exchanger, acting in tandem, play a major role in regulating not only $[\text{Ca}^{2+}]_{\text{cyt}}$ but, more importantly, the ER Ca^{2+} stores and cell responsiveness (Blaustein, 1993). These mechanisms appear to be widely distributed in various types of cells and tissues. For example, we have obtained evidence for these mechanisms in neurons and astrocytes, and in vascular smooth muscle cells. This cooperative behavior of the several transport systems (Na^+ pump, Na/Ca exchanger, and sarco/endoplasmic reticulum Ca^{2+} pump or SERCA pump) is well documented in the heart, where it accounts for the direct cardiotoxic action of cardiac glycosides. It also appears to be involved in the α -adrenergic relaxation of amphibian smooth muscle. We have suggested that it plays a critical role in the local control of blood pressure (i.e., at the level of the vascular

smooth muscle cells and sympathetic nerve endings; Blaustein, 1993).

In immunoblotting experiments, polyclonal antibodies raised against the dog heart exchanger, crossreacted specifically with the exchanger from rat brain synaptic plasma membranes (SPM) and from cultured rat brain astrocyte membranes. The major band was 120 kDa. The amount of exchanger in the SPM approached that in cardiac sarcolemma, whereas that in the astrocyte membranes was considerably lower. In the heart, where Ca^{2+} influx from the ECF is important for initiating contraction, the exchanger plays a major role in Ca^{2+} extrusion. At presynaptic terminals, Ca^{2+} influx is critical for triggering neurotransmitter secretion. Here, too, the exchanger may be the main mechanism for extruding Ca^{2+} following periods of activity.

To examine the role of the Na/Ca exchanger in regulating $[\text{Ca}^{2+}]_{\text{cyt}}$ and ER Ca^{2+} stores in the nervous system, we carried out immunocytochemical studies on cultured neurons and astrocytes, as well as tracer flux and fura-2 studies. The distribution of the plasmalemmal Na/Ca exchanger was investigated in neurons and astrocytes with antibodies raised against the dog heart Na/Ca exchanger. There is a very dense distribution of immunofluorescent labeling at presynaptic nerve terminals. The exchanger is more sparsely distributed on the cell soma, axons, dendrites and growth cones; in all of these portions of the neurons, the immunolabeling occurs in small, discrete patches. This suggests that the role of the exchanger may be somewhat different at presynaptic terminals and in other parts of the neurons. In astrocytes, the labeling also is distributed in discrete patches, but these patches are arrayed in a distinct reticular pattern. This pattern of labeling parallels the distribution of underlying ER that is closely apposed to the plasmalemma (subplasmalemmal or "junctional" ER), as demonstrated with the fluorochrome, DiOC₆(3), that labels ER and mitochondria, and with anti-SERCA-2b antibodies, that label the ER Ca^{2+} pump. These structural observations imply that there is a close functional relationship between the plasmalemmal Na/Ca exchanger and the ER in these cells. Below, we discuss the possibility that a major role of the exchanger may be to regulate the storage of Ca^{2+} in the ER.

The physiological properties of the Na/Ca exchanger in isolated rat brain presynaptic terminals (synaptosomes) were investigated with tracer flux methods. The key observation is that the capacity of the exchanger (i.e., the maximum Ca^{2+} flux mediated by the exchanger) in synaptosomes is surprisingly large: about 2 nmoles Ca^{2+} /mg protein x sec (or ≈ 500 μ moles Ca^{2+} /liter cell water per sec). This is sufficient to exchange about 1/4 of the total nerve terminal Ca^{2+} in 1 sec. This high rate of exchanger-mediated flux correlates with the aforementioned high density of exchangers at nerve terminals.

The function of the Na/Ca exchanger in cultured astrocytes and neurons was studied with fura-2 and digital imaging methods. The key observation was that pretreatment of the cells with 1 mM ouabain, to inhibit the Na^+ pump and raise intracellular Na^+ , greatly augmented the Ca^{2+} transients (transient rises in $[\text{Ca}^{2+}]_{\text{cyt}}$) evoked by glutamate in both types of cells. These augmented responses could be attributed either to increases in the releasable stores of Ca^{2+} in the ER (as a result of altered Na/Ca exchange), or to increased Ca^{2+} entry during the application of the stimulus.

In view of the aforementioned spatial relationship between the plasmalemmal Na/Ca exchanger and junctional ER, we examined the effect of Na^+ pump inhibition on ER Ca^{2+} stores in astrocytes and neurons. In most experiments, the amount of Ca^{2+} stored in the ER was stimulated indirectly, by determining the magnitude of the Ca^{2+} transients evoked by blocking the SERCA with cyclopiazonic acid (CPA) or thapsigargin (TG). CPA and TG both block the same ER Ca^{2+} pump, and deplete the IP₃-releasable Ca^{2+} store, but CPA is reversible whereas

TG is not. The CPA-evoked Ca^{2+} transients were measured in the absence of external Ca^{2+} and Na^+ (removed immediately before application of CPA) to eliminate Ca^{2+} influx and to inhibit Ca^{2+} extrusion via the Na/Ca exchanger.

Fig. 1 shows a representative experiment and illustrates the protocol. Resting $[\text{Ca}^{2+}]_{\text{cyt}}$ was about 100 nM in both neurons (open circles) and astrocytes (solid circles). In Na^+ , Ca^{2+} -free media, 5 μM CPA (a maximal dose) induced a small rise in neuronal soma $[\text{Ca}^{2+}]_{\text{cyt}}$, and a much larger Ca^{2+} transient in astrocytes; the effects were reversible and repeatable. When the cells were incubated with 1 mM ouabain for 12 min, resting $[\text{Ca}^{2+}]_{\text{cyt}}$ rose only slightly in both the neurons and astrocytes (on average, from 114 to 199 nM in astrocytes). However, when CPA was then reapplied, much larger Ca^{2+} transients were observed in both astrocytes and neurons. Furthermore, the cells recovered and again exhibited smaller Ca^{2+} transients when the ouabain was washed out (Fig. 1). The differences in the relative responses of the two cell types raises the possibility that the astrocytes have much larger CPA-sensitive Ca^{2+} stores than do the neuronal somata. This may indicate that Ca^{2+} influx plays a greater role in Ca^{2+} -mediated responses in the neurons, while Ca^{2+} release from ER stores may be more important in the responses of astrocytes.

The mechanism responsible for the ouabain-induced amplification of the CPA response was explored in astrocytes. Inhibition of the Na^+ pump by removal of extracellular K^+ also markedly and reversibly augmented the response to CPA. Thus, the augmentation was not simply due to the ouabain itself. When ouabain was applied in Na^+ -free media, the augmentation was abolished. This implies that the effect of Na^+ pump inhibition was due to the rise in the cytosolic Na^+ concentration, $[\text{Na}^+]_{\text{cyt}}$.

Na^+ pump inhibition is also associated with a loss of K^+ and with cell depolarization. However, the augmented responses to CPA did not appear to result primarily from increased Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels. The depolarization-evoked, external Ca^{2+} -dependent Ca^{2+} transient was abolished by verapamil, but verapamil had negligible effect on the ouabain-dependent augmentation of the CPA-evoked response.

In experiments with the Na^+ -sensitive fluorochrome, SBFI, both 1 mM ouabain and K^+ removal raised $[\text{Na}^+]_{\text{cyt}}$ from 3.3 to 14.6 and 12.5 mM, respectively, in 12 min. These large reductions in the Na^+ electrochemical gradient would be expected to increase $[\text{Ca}^{2+}]_{\text{cyt}}$ markedly as a result of Na/Ca exchanger activity (reduced Ca^{2+} efflux and increased Ca^{2+} influx). Nevertheless, as noted above, only small increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ were observed. Thus, the marked

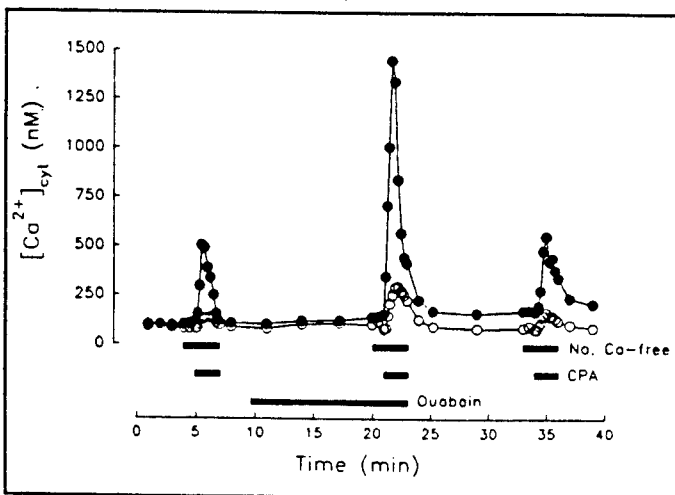


Fig. 1. Effect of incubation with 1 mM ouabain on 5 μM CPA-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ rise (in Na^+ , Ca^{2+} -free medium) in mouse hippocampal neuron/astrocyte co-culture. Bars at bottom indicate solution additions or deletions. Representative data from a neuron (\circ) and an astrocyte (\bullet).

augmentation of the CPA-evoked responses suggests that the cells actually did gain considerable amounts of Ca^{2+} , but that most of it was buffered and sequestered in the ER.

Caffeine releases Ca^{2+} from ryanodine-sensitive intracellular Ca^{2+} stores in many types of cells. In astrocytes, 10 mM caffeine induced a small Ca^{2+} transient, and this, too, was markedly augmented by prior treatment with ouabain. The caffeine-sensitive store is functionally (and probably spatially) distinct from the CPA store in astrocytes because the caffeine-evoked responses were undiminished (and perhaps even augmented) in the presence of CPA.

The ouabain-induced increase in stored Ca^{2+} was directly determined with fura-2 (a Ca^{2+} -sensitive fluorochrome with a much lower affinity than fura-2 for Ca^{2+}) and chlortetracycline (a lipophilic, low-affinity Ca^{2+} sensitive fluorochrome). These dyes, which report primarily on the Ca^{2+} concentrations in membrane-bound stores, confirmed that ouabain increased the Ca^{2+} in the stores, and that the CPA- and caffeine-evoked decreases in stored Ca^{2+} were augmented by ouabain.

In sum, these findings suggest that the ER stores of Ca^{2+} can be modulated by altering the Na^+ electrochemical gradient across the plasmalemma in both neurons and astrocytes. Inhibition of the Na^+ pump raises $[\text{Na}^+]_{\text{cyt}}$; this inhibits Ca^{2+} efflux and increases Ca^{2+} influx mediated by the plasmalemmal Na/Ca exchanger. Large changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ are prevented, however, because most of the intracellular Ca^{2+} is buffered and sequestered in intracellular organelles - especially the ER. The close spatial proximity of the plasmalemmal Na/Ca exchangers and junctional ER suggests that this dynamic regulation of ER Ca^{2+} is an important function of the exchanger. This can be expected to have a profound influence on the responsiveness of cells to agents that activate the cells by promoting the release of Ca^{2+} from the ER (Blaustein, 1993). Furthermore, since disturbed Ca^{2+} homeostasis appears to play a central role in many types of cell injury and cell death, this involvement of the Na/Ca exchanger in the control of ER Ca^{2+} as well as $[\text{Ca}^{2+}]_{\text{cyt}}$ helps to explain why Na^+ accumulation also appears to be a key contributor to these pathophysiological responses (Kiedrowski et al., 1994). The evidence that neurons may have smaller ER Ca^{2+} stores than do astrocytes (Fig. 1) could help to explain why neurons are more vulnerable than astrocytes to toxic insults (Choi et al., 1987).

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AN ANALYSIS OF THE FUNCTION OF THE CHOLINERGIC NERVE TERMINAL: ION CHANNELS, CONFOCAL CALCIUM IMAGES AND OSCILLATIONS. Rami Rahamimoff, Nilly Yakir, Alon Meir, Alexander Butkevitch, Merav Rubin-Fraenkel, Naomi Melamed-Book, Jackie Edry-Schiller, and Simona Ginsburg*. Department of Physiology and The Bernard Katz Minerva Center for Cell Biophysics, Hebrew University Hadassah Medical School, Jerusalem, Israel, and * The Open University, Tel Aviv, Israel.

The understanding of the detailed function of the presynaptic nerve terminal is of prime importance in cholinergic synaptic transmission. We present here the analysis of two major components: ion channels in the nerve ending and the dynamics of intracellular calcium.

Ion channels at the cholinergic nerve endings.

Ion channels play a key role in synaptic transmission. While the channels at the postsynaptic membrane were among the first ion channels to be characterized in physiological, biophysical and molecular terms, relatively little is known about the ion channels at the presynaptic nerve terminal. The main reason for this lack of information is the small size of the nerve terminal and the intracellular structures residing in it, which makes it difficult to apply conventional patch-clamp techniques. In an attempt to overcome this difficulty, a fusion technique was developed (Rahamimoff *et al.*, 1988), which generates large structures suitable for patch clamping. The experiments were performed on the electromotor nerve terminals of *Torpedo ocelata*. Here we describe the ion channels in important presynaptic locations: the surface membrane of cholinergic nerve endings and the membrane of the synaptic vesicle.

Ion channels at the nerve terminal surface membrane.

The release of acetylcholine quanta is regulated by a large variety of cellular and extracellular factors. They include calcium, the frequency of activation of the nerve terminal, hormones and the shape and duration of the action potential. The ion channels present at the surface of the nerve ending can be involved in each one of these regulatory processes.

The surface membrane of the nerve terminal contains a large variety of ion channels (Meir *et al.*, 1993). Among them, three distinct channels were characterized: a bursting potassium channel (Edry-Schiller *et al.*, 1991a), a chloride channel (Edry-Schiller *et al.*, 1991b) and a large non-selective ion channel (Meir and Rahamimoff, 1994).

The potassium channel is a highly selective ion channel. It conducts potassium about 70 fold better than sodium. It belongs to the transient group of ion channels, being activated upon depolarization followed by a very pronounced inactivation. This inactivation is removed by hyperpolarization. The activation and the inactivation properties of this potassium channel, make it a likely candidate to take part in frequency modulation of synaptic transmission. We propose that an action potential invading the nerve terminal causes a release of a certain number of transmitter quanta; it also causes the inactivation of this bursting potassium channel, which is the most abundant channel found in fused nerve terminals. When a subsequent action potential invades the nerve terminal, the number of active potassium channels is reduced due to inactivation, leading to a broadening of the action potential, an augmented entry of calcium and an increased transmitter release.

Statistical memory and oscillations.

A statistical and time series analysis of the bursting potassium channel reveal that it has a memory in the statistical sense. When a large number of depolarizing pulses are given at a constant rate, the response of the membrane patch fluctuates. Some pulses open no channels, other pulses open one, two, or three channels. The number of channels opened by subsequential identical pulses, show a clear statistical memory, which lasts for seconds (Rahamimoff *et al.*, 1992). Thus in addition to the relative fast processes of activation and inactivation, the bursting potassium channel exhibits the slow process of a statistical memory. Under appropriate experimental conditions, molecular oscillations can be observed. Thus, the bursting potassium channel can be a part of the entrainment of synaptic transmission by repeated activation.

Ion channels at the synaptic vesicle.

Ion channels are present not only in the surface membrane of the cholinergic nerve terminal, but also in intracellular organelles. One of the important organelles in quantal transmitter release is the synaptic vesicle. Two types of ion channels were characterized in the fused vesicle membrane: a chloride channel and a non-specific ion channel. The non-specific ion channel conducts equally well cations and anions. It has a very strong voltage dependence. When the membrane patch is kept at around 0mV, this channel is most of the time in an open state. When the membrane is polarized, the channel shows a rapid deactivation, leading to a current rectification. We speculate that the non-specific ion channel may have a physiological role at the various stages of the life cycle of the synaptic vesicle. When the vesicle is an intracellular structure, this channel may be involved in the refilling process. After the fusion of the vesicle with the surface membrane, this channel may contribute to the total conductance of the surface membrane. At the resting potential, due to its voltage dependence, the channel will be mostly in the closed state. But during and after an action potential, the channel will be rapidly activated and thus may take part in frequency modulatory processes in synaptic transmission.

Calcium confocal images.

Calcium ions play a key role in synaptic transmission by regulating the number of transmitter quanta released by the nerve impulse. With the current important developments in fluorescent ion sensitive probes, it is highly desirable to study the calcium transients in the nerve terminal. However, there is a methodological problem. The cholinergic motor nerve terminal at the neuromuscular junction is covered by Schwann cells, which also show changes in their intracellular calcium concentration. To overcome this difficulty, we used calcium confocal microscopy of the nerve terminal of the lizard. This preparation has very large synaptic boutons (Melamed *et al.*, 1993). The terminals were filled with the probe Rhod-2 and image and line scan data were collected. It was found that at rest, the calcium concentration in the synaptic boutons is not constant but shows coordinated fluctuations in the space and in the time domains. In the space domain, very significant correlations were observed among pixels in the same synaptic bouton. This shows that there are waves of calcium in the synaptic bouton. In the time domain, clear oscillations were observed in the intracellular calcium concentration. We do not know whether these oscillations are the origin for the oscillations in transmitter release observed previously after nerve stimulation (Meiri and Rahamimoff, 1978).

Thus, oscillatory processes can be observed at the molecular level in the function of single ion channels, in the intracellular calcium regulation at synaptic boutons, and in the overall activity

of the synapse. It will be of interest to see how these oscillatory processes take part in the coordinated activity of the nervous system.

Acknowledgments

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*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

MONDAY, NOVEMBER 7, 1994

(MORNING, SECOND SESSION)

SYNAPTIC MODULATION

Chair: Dr. D. Fambrough; Co-Chair: Dr. M.T. Shipley

- | | | |
|-------|--------------|--|
| 10:30 | Jahn, R. | Molecular Mechanisms of Synaptic Vesicle Docking and Fusion |
| 11:00 | Llinás, R. | The Role of Calcium Concentration Microdomains in Neuronal Integration and Synaptic Transmission |
| 11:30 | Dolly, J.O. | Neuronal Voltage-Sensitive K ⁺ Channels: Structural and Functional Properties |
| 12:00 | Südhof, T.C. | Molecular Mechanisms Regulating Neurotransmitter Release |
| 12:30 | -- | Lunch Break |

MOLECULAR MECHANISMS OF SYNAPTIC VESICLE DOCKING AND FUSION

Reinhard Jahn, Juan Blasi, Dieter Bruns, Edwin R. Chapman, Lambert Edelmann, Egenhard Link, Christiane Walch-Solimena

Howard Hughes Medical Institute and Departments of Pharmacology and Cell Biology, Yale University School of Medicine, New Haven, CT 06510, USA

Nerve cells, or neurons, transmit information by the release of small molecules, the neurotransmitters. In the resting neuron, neurotransmitters are stored in small membrane-enclosed organelles, the synaptic vesicles. Upon excitation, Ca^{2+} -ions enter the cell and trigger the fusion of the synaptic vesicle with the surrounding plasma membrane (exocytosis), releasing the neurotransmitter molecules into the extracellular space. This process is extremely rapid, with a delay time of only $200\mu\text{s}$. The neurotransmitters activate receptors in the membrane of the receiving cell which leads to a change in their functional status, e.g. to the generation of an electrical signal.

We are interested in the molecular mechanisms which are responsible for synaptic vesicle exocytosis and recycling. During the last few years, we have tried to understand how certain potent neurotoxins inhibit this process. These neurotoxins include tetanus toxin and botulinum toxins which are responsible for the clinical manifestations of tetanus infection or botulism. The neurotoxins are released by bacteria upon ingestion (botulinal toxins only) or upon colonization of wounds. Upon reaching the bloodstream, the toxins bind with high selectivity to motoneurons where they are internalized. Botulinum toxins remain at the periphery and paralyze neuronal excitation of striated muscles, resulting in flaccid paralysis. This feature has led to widespread clinical applications of some of the botulinal toxins in the treatment of local, involuntary muscle spasms, with good success and long-term compatibility. In contrast, tetanus toxin is transported into the central nervous system where it blocks inhibitory interneurons, resulting in overexcitation and cramps.

Tetanus and botulinum toxins are proteins that are produced by bacteria of the genus *Clostridium*. The proteins are dimeric, consisting of a heavy and a light chain that are connected by a disulfide bond. Seven different botulinal toxins exist (named A, B, C1, D, E, F, and G) which are homologous to each other and to the single toxin produced by tetanus bacteria. Research over the last years has revealed that the heavy chain is responsible for binding to nerve endings and for the internalization of the toxins. Inside the synapse, the light chains are released from the heavy chain and cause a potent and long-lasting inhibition of neurotransmitter release. This inhibition is due to a block of exocytosis itself since no other parameters of synaptic function are affected such as membrane potential or Ca^{2+} -influx. Apparently, a single toxin

molecule is sufficient to block transmission of an entire presynaptic nerve terminal, explaining why botulinum toxins are the most toxic compounds known on earth.

Until recently, the mechanisms by which the toxins exert their action were not understood. Some features of the amino acid sequence in the toxin light chains suggested that they may operate as metalloproteases that attack proteins important for exocytosis of synaptic vesicles. In collaboration with Heiner Niemann (Tübingen, Germany), we have confirmed that the toxins are metalloproteases of high specificity. In addition, we have identified the target proteins of all clostridial neurotoxins. Each individual neurotoxin is highly selective for a single protein (for review see Niemann et al., 1994). The targets include the synaptic vesicle protein synaptobrevin (VAMP) and the plasmalemmal proteins syntaxin and SNAP-25. Synaptobrevin is cleaved by the majority of the toxins (tetanus toxin and the botulinum toxins type B, D, F, and G). Each of the toxins cleaves at a single site although the sites are not identical for individual toxins of this group. Cleavage is observed in functionally responding neurons poisoned by the toxins as well as *in vitro* using recombinant synaptobrevin and purified toxin light chains. These findings show that the toxin light chains directly interact with their substrates and do not require additional intermediate steps or cofactors. SNAP-25 is proteolyzed by the botulinum toxins type A and E. Both toxins cleave close to the C-terminus of the protein, releasing fragments of 9 and 26 amino acids, respectively. Syntaxin is cleaved by botulinum toxin type C1. The cleavage site is not yet known but it is close to the membrane anchor domain at the C terminus since toxin cleavage releases a large soluble fragment. In contrast to cleavage of the other proteins, cleavage of syntaxin requires the protein to be anchored to a membrane. The reason for this difference is currently under investigation.

Our findings directly implicate these proteins in a late step in the sequence of events leading to exocytosis of synaptic vesicles. A similar conclusion was also reached by J. E. Rothman and coworkers who have identified the same proteins as membrane receptors for a set of proteins, termed NSF and SNAPs, that are required for intracellular fusion events (Söllner et al., 1993a, 1993b). Thus it appears that these proteins form the core of a conserved multiprotein complex that mediates exocytosis through a series of regulated protein-protein interactions. These interactions are currently under study with the aim of reconstructing individual steps *in vitro* using purified or recombinant protein. Preliminary results indicate that the N-terminal domain of SNAP-25 is required for the binding of SNAP-25 to syntaxin whereas the domain of syntaxin required for this interaction is in the C-terminal portion. Interestingly, both binding domains are likely to be in an α -helical conformation and have a very high propensity to participate in coiled-coil interactions. Furthermore, recombinant SNAP-25 also binds to synaptobrevin. Thus it appears that the vesicle protein synaptobrevin and the plasma membrane proteins syntaxin and SNAP-25 have a high intrinsic affinity for each other. We speculate that these proteins are controlled by

other proteins that allow the formation of a prefusion complex only when synaptic vesicles dock at the active zone. Preliminary results indicate that for synaptobrevin such control is exerted by partner proteins present in the synaptic vesicle membrane.

Shortening of the C-terminus of SNAP-25 by 9 amino acids does not affect its binding to syntaxin but weakens the interaction with synaptobrevin. This suggests that cleavage of SNAP-25 by BoNT/A selectively interferes with the binding of these two proteins, thus inhibiting exocytosis. Additional, albeit indirect evidence for SNAP-25 as the protein solely responsible for the toxic effect of BoNT/A has been obtained in our recent studies on cultured synaptic preparations. Using the leech Retzius-P cell pair as a model system for fast synaptic transmission (Bruns et al., 1993), we have shown that microinjection of clostridial neurotoxin L-chains effectively blocks synaptic transmission. This was no surprise since all substrate proteins are highly conserved in evolution and since the neurotoxins were previously shown by others to be effective in invertebrate neurons (Poulain et al., 1993). However, BoNT/A failed to have any effects on synaptic transmission. Cloning of leech SNAP-25 revealed that, despite a high degree of sequence conservation, the protein carries several amino acid substitutions at the C-terminal end. We then tested whether leech SNAP-25 can still be cleaved by BoNT/A or BoNT/E. The results clearly show that this is not the case, indicating that the resistance of the Retzius cell to BoNT/A and E is due to the toxin resistance of SNAP-25. We are currently using microinjection of recombinant fragments of SNAP-25 as well as SNAP-25 antibodies to perturb synaptic transmission. Our preliminary results are in accord with the view that this protein is an essential component of the exocytotic fusion machine.

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THE ROLE OF CALCIUM CONCENTRATION MICRODOMAINS IN NEURONAL INTEGRATION AND SYNAPTIC TRANSMISSION

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An emerging concept in biology seems to be that calcium-concentration changes in the immediate interior of the cell membrane—following activation of voltage- or ligand-dependent channels—occur within a very specialized volume that is strictly related to the functioning of calcium as a second messenger. Indeed, measurements concerning the distribution of the calcium-concentration profile, and the very speed at which calcium entry is capable of activating such events as synaptic release or activation of calcium-dependent ionic conductances, argue for a highly organized spatial distribution of intracellular organelles, with regard to calcium concentration.

Examples will be given of the relation between calcium-microdomain localization as established with n-aequorin-J, and the actual site of transmitter release that is known to be a synaptic activation zone for squid giant synapse. Examples from other laboratories have offered similar evidence concerning activation of calcium-dependent potassium channels. In addition, histochemical studies further indicate the possibility that the calcium microdomains may have other residents in place relating to plasmalemmal calcium pumps, as well as the possibility of secondary-calcium release from intracellular stores as, for instance, in subcisternal neurons in regard to IP₃-dependent intracellular release from calcium stores.

NEURONAL VOLTAGE-SENSITIVE K⁺ CHANNELS:
STRUCTURAL AND FUNCTIONAL PROPERTIESDolly, J.O.Department of Biochemistry, Imperial College of Science
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Voltage-sensitive K⁺ channels serve important roles in the nervous system where they act as major determinants of neuronal excitability, influencing the resting membrane potential, wave forms and frequency of action potentials, and thresholds of excitation. Accordingly, the family of fast-activating, aminopyridine-sensitive K⁺ channels consists of several subtypes, as revealed by electrophysiological techniques and through using the selective blockers, α -dendrotoxin (α -DTX) or its homologues from mamba snake venoms. α -DTX has been instrumental in the biochemical identification, localisation in mammalian brain and purification of these channels (Dolly *et al.*, 1994). Also, the abilities of such toxin probes to facilitate transmitter release, induce seizures and cause neurodegeneration in rodent brain are aiding investigations into epileptic mechanisms (Bagetta *et al.*, 1994). Together with the complementary approach of molecular genetics, the structures of these K⁺ channels have, thus, been defined at the oligomeric and subunit levels.

α -DTX - susceptible K⁺ channels are hetero-oligomeric glycoproteins. Following successful purification of α -DTX acceptors from bovine cerebrocortical membranes, sedimentation analysis revealed that these native K⁺ channels are large sialoglycoproteins ($M_r \sim 400$ kD) composed of α and β subunits. Isoforms of each of the latter were found and a stoichiometry of (α)₄(β)₄ has been deduced (Dolly *et al.*, 1994).

Primary structure and functional domains of the α subunits. N-terminal microsequencing of the major α subunit isoform comprising the α -DTX acceptors allowed cloning of its gene from bovine brain that showed it to be virtually identical to one (K_v1.2 or α 5) cloned from rat cerebral cortex, using a *Drosophila* Shaker probe (reviewed by Dolly *et al.*, 1994). Several distinct α subunit genes have been isolated by numerous groups and expression of each yields a characteristic K⁺ current, some of which are blocked by α -DTX. All of these proteins are speculated to contain 6 α helical membrane-spanning regions plus a β -hairpin loop that traverses the bilayer and houses the K⁺ filter. Importantly, residues essential for binding of the toxins are strategically located near the outer mouth of the filter.

α Subunit isoforms in K^+ channel oligomers. Probes specific for each of several α subunits were provided by monoclonal antibodies, raised against the above-mentioned purified K^+ channels, and antisera produced with fusion proteins containing distinct C-terminal regions of the different subunits (Scott *et al.*, 1994). After purification, these IgG preparations were shown to be α isoform - specific. Western blotting of the pure K^+ channel preparation revealed the presence of 4 distinct α ($K_v1.1$, 2, 4 and 6), plus β , subunits. Most notably, immunoprecipitation and cross-blotting experiments demonstrated that oligomeric K^+ channel complexes can contain combinations of many α -isoforms, together with the tightly-associated β subunits. These findings, together with reports that co-expression of two α -isoforms yields a distinct K^+ current, emphasise that diversity can arise by hetero-oligomeric assembly of different α -isoforms with the β subunits. A discrete presynaptic location for a K^+ channel containing the $K_v1.2$ α subunit has been established immunologically in the GABAergic nerve terminals of basket cells in rat cerebellum where it could act to control excitability of adjacent Purkinje cells (McNamara *et al.*, 1993).

β Subunit variants: cloning and structural properties. Micro-sequencing of enzymically-produced peptides from the isolated β subunit gave adequate information to design oligonucleotide probes for successful cloning of the encoding gene, β_2 , from bovine cortex (Scott *et al.*, 1994a). The deduced amino acid sequence is unique and gives a predicted protein size of 40,983D, a value close to that found by SDS-PAGE for the native β subunit. In contrast to the α subunits, no membrane-spanning segments were detected. In fact, the β subunit has been speculated to reside on the cytoplasmic side of the membrane in tight association with the α subunits because of its hydrophilic nature, lack of N-glycosylation sites and a leader sequence plus the presence of numerous consensus sites for kinases (consistent with our demonstrated phosphorylation of the β subunit in the native K^+ channels). An equivalent β_2 sequence was cloned from rat brain but, in addition, a highly homologous β_1 subunit was obtained except for having a divergence and an extension (34 residues) at its N-terminus (Rettig *et al.*, 1994).

Co-expression of β_1 with α subunits accelerates K^+ current inactivation. Although expression of β_1 mRNA in *Xenopus* oocytes failed to produce a detectable K^+ current, its co-expression with that for $K_v1.1$ α subunit dramatically shortened the inactivation time; in essence, there was a conversion from a sustained time course typical of a delayed-rectifier to that resembling an A-type K^+ current (Rettig *et al.*, 1994). β_1 also accelerated the inactivation of the K^+ current produced by $K_v1.4$, an effect that persisted even after N-type inactivation of the $K_v1.4$ K^+ current was removed by deleting 110 residues from its N-terminus. The observed abolition of this effect

of β_1 by oxidation conditions could be prevented by mutation of a cysteine at position 7 to serine. Lastly, truncation of 34 N-terminal residues from β_1 removed its influence on K^+ channel inactivation whereas addition to isolated membrane patches of a 24-residue synthetic peptide, corresponding to the N-terminus of β_1 subunit, restored its effect on K^+ current inactivation. The smaller β_2 subunit, that lacks the latter N-terminal sequence, failed to affect the inactivation time courses of the K^+ channels tested. Thus, it is concluded that the β_1 , but not β_2 , subunit possesses a cysteine-containing domain in its N-terminus that speeds up K^+ current inactivation, an effect mimicked by the non-covalently attached peptide. The presence or absence of a β_1 subunit provides another means of creating K^+ channel diversity, allowing their biophysical properties to be optimised for the role served in any given neuronal environment.

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MOLECULAR MECHANISMS REGULATING NEUROTRANSMITTER RELEASE

Thomas C. Südhof

Abstract for the International Symposium on the Cholinergic Synapse:
Structure, Function, and Regulation, November 6-10, 1994.

When an action potential reaches a presynaptic nerve terminal, Ca^{2+} flows into the terminal via voltage-gated Ca^{2+} channels and triggers neurotransmitter release. In resting nerve terminals, neurotransmitters are stored in synaptic vesicles, abundant secretory organelles of uniformly small size. Ca^{2+} triggers neurotransmitter release by stimulating synaptic vesicle exocytosis. After exocytosis, empty synaptic vesicles rapidly re-endocytose and refill with neurotransmitter. Synaptic vesicles are relatively simple organelles whose only known function resides in their role in neurotransmission. Because of their central role in this process, they have been intensely studied in recent years, leading to a nearly complete description of their intrinsic membrane components (reviewed in Jahn and Südhof, 1993).

Ca^{2+} triggering of neurotransmitter release is very complex. It is thought that at a given synapse, Ca^{2+} acts by increasing the probability of release. According to this model, the spontaneous release activity observed in the absence of stimulation (the so-called miniature endplate potentials at a neuromuscular junction) is due to the finite probability of exocytosis at resting Ca^{2+} concentrations. Even in stimulated synapses, the release probability rarely reaches a value of 1, resulting in most synapses experiencing less than one vesicle exocytosis per stimulation. In addition, recent experiments have shown that different synapses can have dramatically different release probabilities. One of the possible mechanisms by which the release probability could be modulated is by a modulation of the release apparatus. This mechanism would basically lead to a change in the probability of synaptic vesicle exocytosis as a function of Ca^{2+} .

In electron micrographs of resting nerve terminals, a multitude of synaptic vesicles can usually be observed in a docked state at the active zone of the synapse (Peters et al., 1991). This suggests that the supply of synaptic vesicles for release is not physiologically limiting, and that regulatory events have to operate on docked vesicles and not on the recruitment of vesicles to the docked stage. The modulation of synaptic neurotransmission by previous synaptic activity is usually referred to as synaptic plasticity. Many events of synaptic plasticity are Ca^{2+} dependent and change the probability of neurotransmitter release (reviewed in Zucker, 1989). Together these observations suggest that Ca^{2+} has a dual regulatory action on a synapse: It acutely triggers release by stimulating synaptic vesicle exocytosis, and on a longer time course it modulates release probability, possibly by protein kinases that enhance or decrease the probability of exocytosis of docked vesicles.

My laboratory is interested in elucidating molecular mechanisms that are involved in Ca^{2+} -regulated steps in the synaptic vesicle pathway. We first identify and characterize molecular components with a putative role in these processes, and then test their physiological functions by a variety of methods. These studies have led to the identification of synaptotagmins I and II, intrinsic membrane proteins of synaptic vesicles, as likely major Ca^{2+} sensors for synaptic vesicle exocytosis (Perin et al., 1990; Davletov and Südhof, 1993). Synaptotagmins I and II are prime candidates for a role as Ca^{2+} sensors because of their high abundance and evolutionary conservation, their localization on the synaptic vesicles which would be exactly where the Ca^{2+} sensor has to act, and their Ca^{2+} -binding properties which include a weak activation by Sr^{2+} and Ba^{2+} and a cooperativity of binding which matches that of release (Dodge and Rahamimoff, 1967). However, there has been considerable controversy regarding the function of synaptotagmins in recent years, some of which may have been caused by the multifunctional properties of this protein family that are now beginning to be recognized. Recent experiments in mutant mice, however, have suggested that synaptotagmin I is indeed the major Ca^{2+} sensor for release in some central synapses (Geppert et al., 1994 [submitted]). Furthermore, a possible function for synaptotagmins in endocytosis was demonstrated with the discovery that synaptotagmin I is a high

affinity receptor for AP2, a protein complex that mediates the recruitment of clathrin to membranes (Zhang et al., 1994).

In a different set of experiments, we have tried to determine the functions of other conserved components of the synaptic vesicle pathway that might function in the Ca^{2+} -dependent modulation of release probability. One of the most interesting proteins of synaptic vesicles is synapsin I, originally discovered by P. Greengard's laboratory as one of the major protein kinase substrates in brain. Synapsin I contains phosphorylation sites for at least four kinases and binds to microtubules, spectrin, actin microfilaments, and neurofilaments, with the last two binding activities being regulated by Ca^{2+} , calmodulin-dependent protein kinase II (reviewed in Jahn and Südhof, 1993; Greengard et al., 1993). The multiple phosphorylation events on synapsin I make it one of the prime candidates for a regulatory role in neurotransmission. Such a function could be detected in knock-out mice lacking synapsin I which showed an alteration in short term plasticity, suggesting that synapsin I has an essential function in regulating the probability of synaptic vesicle exocytosis at the active zone (Rosahl et al., 1993). However, synapsin I is a member of a protein family that also includes synapsin II (Südhof et al., 1989), and future experiments will have to elucidate what additional functions synapsin I has that it redundantly shares with synapsin II.

As discussed above, the effects of Ca^{2+} on synaptic vesicle functions is likely to be complex. We are now only at the beginning of an understanding of the molecular events underlying synaptic regulation. Fundamentally, two types of regulatory mechanisms by which Ca^{2+} could act can be envisioned: Binding of Ca^{2+} to synaptic Ca^{2+} -binding sites proteins which results in a direct regulatory effect, and indirect effects by activation of protein kinases. Already now there is a host of proteins with a likely function in synaptic vesicle traffic that are either substrates for Ca^{2+} -activated kinases and/or Ca^{2+} binding proteins. For example, in addition to synaptotagmins, synaptic vesicles also contain rabphilin-3A, a likely Ca^{2+} binding protein that also binds to rab3A. Recent experiments with mice lacking rab3A have demonstrated that these mice are deficient in synaptic vesicle recruiting during repetitive stimulation and that they have decreased

levels of rabphilin-3A, suggesting that rabphilin-3A may have an effector function for rab3A in synaptic vesicle docking (Geppert et al., 1994). In addition to being a Ca^{2+} -binding protein, rabphilin-3A may also be substrate for Ca^{2+} -activated kinases. These observations raise the possibility that synaptic vesicle docking may also be regulated by Ca^{2+} via rabphilin-3A. Future experiments will have to elucidate how this potential mechanism may work, and what the functions of the many other synaptic Ca^{2+} binding proteins and kinase substrates are.

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*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

NOTES

MONDAY, NOVEMBER 7, 1994

(AFTERNOON, FIRST SESSION)

SYNAPTIC MODULATION AND PHOSPHORYLATION

Chair: Dr. P. Greengard; Co-Chair: Dr. G. Inesi

- | | | |
|-------|---------------|--|
| 13:30 | Westbrook, G. | Regulation of Glutamate Channels at Central Synapses |
| 14:00 | Tsien, R. | Ca ²⁺ Channels in CNS Neurons and Neurotransmission at the Level of Single Synaptic Boutons |
| 14:30 | Dani, J. | Calcium Modulation and Calcium Permeability of Nicotinic Acetylcholine Receptors |
| 15:00 | -- | Coffee Break |

REGULATION OF GLUTAMATE CHANNELS AT CENTRAL SYNAPSES

Westbrook, Gary L.

Vollum Institute, Oregon Health Sciences University Portland OR USA

Overview

Studies of cholinergic synapses have provided much of our basic knowledge of ligand-gated channel function as well as many fundamental aspects of synaptic transmission. For many years, the difficulty in performing analogous experiments at central excitatory synapses led to the default hypothesis that central synapses were essentially 'clones' of the neuromuscular junction. As studies of central excitatory synapses have accelerated in recent years, these experiments have revealed, if not totally new concepts, at least interesting variations on prior themes. Prominent among the differences are the wide range of glutamate receptor subunits and metabotropic receptor subtypes that contribute to these synapses, the apparently prominent roles for desensitization and transporters in terminating transmitter action, and the striking variety of modulatory influences on postsynaptic glutamate channels (1). The last category includes effects of kinases, phosphatases and allosteric modulators. I would like to add to this list of modulators by discussing some of our experiments that suggest a dynamic role for certain cytoskeletal proteins in regulating glutamate channel function.

As with other aspects of synaptic transmission, the idea that cytoskeletal proteins are important in synaptic transmission has its roots at the neuromuscular junction. However the main effect at the neuromuscular junction appears to be primarily structural, e.g. the 43 kD protein is closely associated with the acetylcholine receptor and is involved in the induction and/or maintenance of postsynaptic receptor clusters. In the first set of experiments, I will review experiments examining the calcium-dependent regulation of N-methyl-D-aspartate (NMDA) channels and the possible role of the actin cytoskeleton in mediating this effect. In the second set of experiments, we have examined the action of a different class of cytoskeletal proteins, the so-called A-kinase anchoring proteins (AKAPs), on regulation of the AMPA receptors. All experiments were conducted in cultured rat hippocampal neurons using standard patch clamp methods.

NMDA channel regulation and the actin cytoskeleton

The special role assigned to NMDA channels in excitatory neurotransmission is based on their voltage-dependence, relatively high calcium permeability as well as channel kinetics which give rise a much slower synaptic response compared to the fast EPSC mediated by AMPA receptors. Like many channels that display calcium permeability, NMDA receptors are themselves regulated by increases in intracellular calcium. In whole-cell experiments, increases in intracellular calcium reduce NMDA currents in two separate experimental paradigms. In the first, 3-5 second applications of agonist result in a slow current relaxation that decays to a steady state value of approximately 50% of the initial amplitude. The time constant of the relaxation is approximately 1-2 sec in 2 mM extracellular calcium with the pipette pipette solution buffered with 10 mM EGTA. The decay rate is due to the rate of calcium accumulation in the cell rather than to the kinetics of the underlying Ca-dependent process. The reduction in NMDA current can also be induced by flash

photolysis of 'caged' Ca or a voltage step to activate voltage-dependent calcium channels with time constants of less than 100 ms. Because the receptor need not be activated for down regulation to occur, we have termed this phenomena 'inactivation' to distinguish it from other receptor-mediated forms of desensitization, specifically glycine-dependent and -independent desensitization. The reduction in current is due to a reduction in the open probability of NMDA channels, and does not represent two populations of channels only 50% of which are calcium-sensitive.

In the second paradigm, whole cell recording in the absence of ATP in the patch pipette leads to loss of approximately 50% of the initial current as well as loss of the Ca-dependent relaxation (i.e. inactivation). The time-dependent current loss or 'rundown' is also completely Ca-dependent. That is, if the agonist solution is Ca-free, no loss of current is observed even in the absence of ATP. Of note, exogenous ATP maintains the peak current but does not prevent inactivation suggesting that a two-stage mechanism is necessary to explain the action of intracellular calcium on the NMDA channel. As neither inactivation or rundown were affected by pharmacological manipulation of kinases or phosphatases, we considered other possible mechanisms. We found that the polymerization state of the actin cytoskeletal can account for the second stage of calcium-dependent regulation.(2). This is based on the ability of phalloidin, a mushroom toxin that specifically inhibits the depolymerization of actin, to block rundown even in the absence of exogenous ATP whereas cytochalasin D, which destabilizes actin filaments, induces a reduction in the NMDA current similar to that observed in the absence of exogenous ATP. Like ATP, phalloidin does not block the first component, i.e. inactivation. Taxol and colchicine which stabilize and destabilize microtubules are without effect.

These results lead to two basic questions: 1. Is this regulatory mechanism operable in synaptic transmission; and 2. what are the molecular elements that underlie it. The answer to the first question appears to be yes. For excitatory postsynaptic currents (EPSCs) in cultured hippocampal neurons, a whole cell prepulse of NMDA in Ca-containing medium depresses a subsequent test pulse of NMDA as well as the evoked NMDA receptor-mediated EPSC while AMPA receptor-mediated EPSCs are unaffected. This demonstrates that the subsynaptic NMDA receptors are down regulated by intracellular calcium. Likewise regular stimulation of NMDA receptor mediated EPSCs in the absence of exogenous ATP leads to a selective decrease in the NMDA currents, suggesting that Ca influx through NMDA receptors is also sufficient to cause down regulation. It remains to be resolved whether calcium influx at a single release site is sufficient to influence the open probability of NMDA receptors at that synapse.

With regard to the second question, biochemical and molecular confirmation of our model is lacking. The lack of Ca-sensitivity in inside-out patches might suggest that the calcium sensor is not an intrinsic part of the receptor, but rather resides on a cytoplasmic regulator protein, perhaps analogous to the β subunits of voltage-dependent calcium and potassium channels. This putative regulatory protein could also be one of the known Ca-dependent actin-binding proteins. We have also not yet tested whether Ca/CaM might be sufficient to restore Ca-sensitivity to channels in excised patches. Finally an intriguing possibility is that the large intracellular C-terminal domain of the NR2 subunits (most likely 2A or 2B in the case of cultured hippocampal neurons) could itself function as a Ca-dependent ball-and-chain mechanism, interacting both with the channel gate and with the underlying cytoskeleton.

Anchoring of protein kinase A and AMPA receptor function

A second way in which cytoskeletal proteins could affect synaptic receptor function is by localizing regulatory proteins such as kinases near their substrates. Recently a family of proteins which bind the regulatory subunit (RII) of protein kinase A (PKA) have been characterized. These proteins have been postulated to anchor kinases to the plasma membrane as well as internal membranes, and thus have been called A kinase anchoring proteins (AKAPs). Because AMPA receptor activity is regulated by PKA, we tested whether the endogenous kinase must be anchored to regulate AMPA receptors in cultured hippocampal neurons. We made use of the known RII binding domain of the AKAPs to generate peptides which could compete for RII with endogenous AKAPs. Whole cell currents evoked by kainate or AMPA have a constant amplitude during 25-30 minutes of recording. However, intracellular dialysis with PKI, a specific inhibitor of the catalytic subunit of PKA, reduces the current to approximately 60% of control with a time constant of 5-10 minutes. Dialysis with the inhibitor peptide produced a similar inhibition which was non-additive with PKI. A control peptide, derived from the same binding sequence but which does not bind RII, had no effect. Consistent with the idea that the anchoring inhibitor peptide displaces the PKA holoenzyme from the membrane, addition of purified catalytic subunit overcame the effect of the peptide. Experiments examining spontaneous AMPA-mediated synaptic currents produced similar results (3).

Summary

These two sets of experiments suggest that structural elements can play an active role in the regulation of synaptic receptors. Although this may seem surprising, one only needs consider the complex meshwork of cytoskeletal elements in the postsynaptic density and the high concentrations of actin in the dendritic spines to at least consider this as a plausible hypothesis. The rapid calcium-dependent remodeling of the actin cytoskeleton in nerve growth cones also provides a conceptual model suggesting that structural proteins are in fact in very dynamic equilibrium. Such interactions may provide the compartmentalization necessary to facilitate specific interactions between receptors and their regulatory proteins such as kinases and phosphatases.

Acknowledgments

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Ca²⁺ Channels in CNS Neurons and Neurotransmission at the level of Single Synaptic Boutons

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The diversity of Ca²⁺ channels in vertebrate neurons is of fundamental interest because voltage-gated Ca²⁺ entry through these channels controls a wide variety of physiological functions, including neurotransmitter release, membrane excitability, neurite outgrowth, metabolism and gene expression. The existence of more than one type of voltage-gated calcium channel in vertebrate neurons has been appreciated for some time (Carbone and Lux, 1984; Armstrong and Matteson, 1985; Nowycky et al., 1985; Fedulova et al., 1985). Biophysical and pharmacological analysis has led to the description of several classes of Ca²⁺ channels, usually referred to as L-, N-, T- and P-type (Fox et al., 1987a,b; Llinas et al., 1989; Bean 1989). These channel types differ in their responsiveness to neuromodulators (Tsien et al., 1988), their distribution among various types of neurones (Miller and Fox, 1990; Tsien et al., 1991; Mintz et al., 1992b; Snutch and Reiner, 1992) and their localization in different regions within individual neurons (Westenbroek et al., 1992). The variety of Ca²⁺ channel types allows many possibilities for multiplicity of function.

The full extent of Ca²⁺ channel diversity remains incompletely understood. On one hand, molecular cloning has uncovered an ever-increasing number of Ca²⁺ channel subunits with a wide distribution in the CNS (Perez-Reyes et al., 1990; Tsien et al., 1991; Snutch and Reiner, 1992; Miller, 1993). On the other hand, pharmacological evidence points to the possible existence of additional types of Ca²⁺ channels outside of the L-, N-, T- and P-type classification (Regan et al., 1991; Artelejo et al., 1992; Mintz et al., 1992b, Swartz et al. 1993). While both lines of investigation suggest additional complexity, the match-up between the cloned subunits and Ca²⁺ channels in neurons is incomplete. Some Ca²⁺ channel α_1 subunits appear in good correspondence with established channel types. For example neuronal L-type channel currents are generated by class C and D α_1 subunits (Mikami et al., 1989; Williams et al., 1992a) and N-type Ca²⁺ channels arise from the class B α_1 (Williams et al., 1992b). Other α_1 's, however, are more difficult to identify as known calcium channel types in nerve cells. A leading example is the class A α_1 subunit (also known as brain-I or BI), the first α_1 subunit to be isolated from nervous tissue (Mori et al., 1991; Starr et al., 1991). Transcripts for α_{1A} abound in the mammalian brain, particularly in cerebellar Purkinje and granule cells (Mori et al., 1991, Fujita et al., 1993). Because the Ca²⁺ current in Purkinje neurons is almost entirely due to the P-type channel (Llinas et al., 1989; Mintz et al., 1992), this has led to the idea that α_{1A} and P-type current are equivalent (Snutch and Reiner, 1992). However, expression of α_{1A} in oocytes generates an inactivating current, different in both kinetics and pharmacology from the non-inactivating P-type current (Sather et al., 1993; Stea et al., 1994). This suggests that α_{1A} may also be capable of generating a novel non-P-type Ca²⁺ channel current.

Dissection of Multiple Types of Ca²⁺ Channel Currents in Rat Cerebellar Granule Neurons

We have studied the diversity of Ca²⁺ channel types in rat cerebellar granule cells. Granule neurons give rise to parallel fiber synapses onto the dendrites of Purkinje neurons, and are the

most abundant neuron in the mammalian CNS. Contributions of five different components of high-voltage activated Ca^{2+} channel current were distinguished with a series of potent channel inhibitors in whole-cell recordings (5 mM external Ba^{2+}). Nimodipine-sensitive L-type current and ω -CTx-GVIA-sensitive N-type current contributed 15% and 20% of the total current respectively. The bulk of the remaining current (46%) was inhibited by ω -Aga-IVA. The current blocked by this toxin was further subdivided into two components, P- and Q-type, on the basis of differences in their inactivation kinetics and sensitivity to ω -Aga-IVA. P-type current was non-inactivating during 0.1 s depolarizations, half-blocked at about 1-3 nM ω -Aga-IVA, and contributed ~11% of the total current; Q-type current was prominently inactivating, half-blocked at ~90 nM ω -Aga-IVA, and comprised 35% of the total current. Both P- and Q-type currents were potently inhibited by ω -CTx-MVIIC. A current component resistant to all of the aforementioned blockers (R-type) displayed more rapid inactivation than the other components and constituted 19% of the total current. The Q-type current, the largest of the current components in the granule neurons, closely resembles the currents that were generated in *Xenopus* oocytes by the expression of the α_{1A} subunit. The overall conclusion is that CNS neurons may display an even greater variety of Ca^{2+} channels than readily imagined a few years ago.

Roles of N-type and Q-type Ca^{2+} Channels in Supporting Hippocampal Synaptic Transmission

In light of this kind of evidence for several pharmacologically distinct high voltage-activated Ca^{2+} channels on neuronal cell bodies, an obvious question is how the diverse Ca^{2+} channels contribute to transmission in the CNS. We studied the synapse between hippocampal CA3 and CA1 neurons (Wheeler et al., 1994), a focus of interest in the examination of glutamatergic transmission and synaptic plasticity. At this synapse, inhibition of L-type channels by nifedipine has little effect, and much of the transmission remains after blockade of N-type channels by ω -CTx-GVIA. We found that the Ca^{2+} channels that mediate the remaining transmission are pharmacologically distinct from classical L-type Ca^{2+} channels and are less sensitive to ω -Aga-IVA than P-type channels described on Purkinje cell bodies (Wheeler et al., 1994). Instead, their pharmacological profile resembled that of α_{1A} Ca^{2+} channel subunits expressed in *Xenopus* oocytes and the Q-type Ca^{2+} channel current in cerebellar granule neurons. Stimulation of neurotransmitter receptors can greatly attenuate synaptic transmission mediated by α_{1A} channels. Reductions in the contribution of either Q- or N-type channels greatly increased the degree to which closely spaced stimuli facilitated synaptic transmission. These results suggest that cooperation may occur among multiple Ca^{2+} channel types in the control of transmitter release and may be advantageous for precise regulation of the strength or frequency-dependence of synaptic function. Our results are compatible with findings from several other groups (Takahashi, Dunlap, Saggau, Nicoll, Laurent) in implicating multiple types of Ca^{2+} channels in the release of various transmitter substances. It will be interesting to see whether this also holds true for cholinergic synapses.

Depolarization-Evoked Transmitter Release from Single Presynaptic Boutons

Several fundamental questions about the nature of synaptic communication between CNS neurons could be answered if it were possible to study transmission at the level of individual synapses on a regular basis. How many transmitter quanta can be released from a bouton before it becomes depleted? How large is the quantal response? How variable is this unitary size within an individual

bouton and from one bouton to the next? To try to answer some of these questions, we have applied a combination of electrical and optical recording methods to study synaptic currents generated by transmitter release from single, visually identified boutons of hippocampal neurons (Liu & Tsien, 1994). Following the approach pioneered by Betz and colleagues at the neuromuscular junction (Betz et al., 1992), functional boutons of cultured hippocampal pyramidal cells (2-3 day postnatal rats, neurons kept in culture 12-30 days, see Malgaroli & Tsien, 1992) were localized by their ability to take up the styryl dye FM-143 (Ryan et al., 1993). This endocytotic uptake was elicited in the wake of depolarization-induced exocytosis (70 mM [K⁺] for 1 min, wash for > 5 min). Global depolarization with 70-90 mM K⁺ caused destaining, an effect blocked by 10 μM Cd²⁺. In the absence of tetrodotoxin, local electrical stimulation evoked EPSCs and also caused FM-143 destaining of a presynaptic axon. In the presence of TTX, focal electrical stimulation caused localized destaining of 1-4 boutons. For most studies, we stimulated one or more boutons by local application of high K⁺ plus 0.5-2 mM Ca²⁺ from a 1 μm puffer pipette, while monitoring excitatory synaptic currents with whole cell recordings from the cell body of the postsynaptic hippocampal neuron. The bulk of the cell was bathed with TTX and 0.2 mM Ca²⁺ to restrict synaptic activity to the locus of stimulation. The local nature of the stimulus was verified by small lateral movements of the stimulating pipette; often, displacements of 2 μm made the difference between effective and non-effective stimulation. Under appropriate conditions, FM-143 destaining was observed for a stimulated bouton but not its neighbors. The incidence of synaptic currents was very low in the absence of stimulation, grew progressively during the high K⁺ pulse, and fell off sharply following its cessation. This is consistent with what would be expected for a K⁺-induced depolarization that gradually reaches steady-state and a steep voltage-dependence of Ca²⁺ channel opening. With repeated high K⁺ pulses (0.6 s every 5 s), the number of EPSCs per trial declined gradually from a peak to a much lower baseline (τ~5-10 pulses), as expected for depletion of the pool of releasable, glutamate-containing vesicles. The number of EPSCs associated with this depletion was typically ~50-100 per stimulated bouton (n=6 cells). A 4-6 min rest period at room temperature was sufficient to achieve complete recovery. This method allows us to examine the heterogeneity in EPSC amplitude among different boutons on the same postsynaptic cell. The variation in median amplitude for synapses onto a given cell was relatively small compared the the variation among different cells. The median size appeared to decrease as the density of innervation increased. This approach is promising as a means of gaining information about kinetic properties of transmitter release and postsynaptic responses at individual synapses.

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CALCIUM MODULATION AND CALCIUM PERMEABILITY OF NICOTINIC ACETYLCHOLINE RECEPTORS

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Nicotinic acetylcholine receptors (nAChRs) form a family of ligand-gated ion channels found at the vertebrate neuromuscular junction and throughout the central nervous system. Based on the polypeptide subunits that form the receptor and the resulting functional properties, nAChRs can be classified into three main categories: muscle nAChRs, neuronal nAChRs, and neuronal α -bungarotoxin (α -BGT) binding proteins. Muscle nAChRs mediate the high efficiency synaptic transmission at the vertebrate neuromuscular junction. Neuronal nAChRs are a more diverse second category consisting of many different subtypes. Genetic diversity in the subunit composition of the neuronal receptor subtypes probably underlies the functional and pharmacological diversity that has been observed. This category of neuronal nAChRs is not inhibited by α -BGT, but there is a third category of nAChRs that is inhibited by α -BGT. The function and complete composition of native neuronal α -BGT receptors is still unknown, but the homo-oligomeric channels formed by $\alpha 7$ in oocytes are inhibited by α -BGT as are some nicotinic currents in the CNS.

Our recent research has differentiated the three categories of nAChRs based on their ability to permeate calcium ions. Although the permeability ratio of Ca^{2+} to Na^+ or Cs^+ does not tell the amount of Ca^{2+} moving through a channel, the ratio is relatively easy to obtain from reversal potential measurements and the ratio can be used to rank the relative Ca^{2+} permeability of the 3 major categories of nAChRs. Based on permeability ratio estimates, muscle nAChRs have a small, but significant, Ca^{2+} permeability ($P_{\text{Ca}}/P_{+} \approx 0.2$). Neuronal nAChRs have a greater Ca^{2+} permeability. Ganglionic nAChRs have a P_{Ca}/P_{+} of about 1.5, and α -BGT-sensitive nAChRs composed of the neuronal $\alpha 7$ subunit have an even greater Ca^{2+} permeability ($P_{\text{Ca}}/P_{\text{Na}} \approx 20$).

Because permeability ratios only provide a relative scale and do not directly indicate Ca^{2+} flux, we developed a new approach to determine quantitatively the fraction of current carried by Ca^{2+} through an ion channel under physiological conditions. This approach entails the simultaneous measurement of membrane current and intracellular Ca^{2+} for single cells. Whole-cell patch-clamp techniques were used to measure current, and intracellular Ca^{2+} was monitored with the fluorescent indicator fura-2. To obtain a quantitative measure of the fraction of current carried by Ca^{2+} , a cell-by-cell calibration method was devised to account for differences among cells in such factors as cellular volume and Ca^{2+} buffering. The method was used to evaluate the Ca^{2+} flux through muscle and neuronal nAChRs. In a solution containing 2.5 mM Ca^{2+} at a holding potential of -50 mV, Ca^{2+} carries 2.0% of the inward current through muscle nAChRs from BC3H1 cells and 4.1% of the inward current through neuronal nAChRs from adrenal chromaffin cells. The Ca^{2+} flux through neuronal nAChRs of adrenal chromaffin cells is insensitive to α -bungarotoxin. The influx of Ca^{2+} is voltage dependent, and because of the Ca^{2+} concentration difference across the cellular membrane, there is Ca^{2+} influx into the cell even when there is a large net outward current.

The Ca^{2+} permeability of nAChRs is of interest because synaptic activity can generate intracellular and extracellular Ca^{2+} signals that influence cellular excitability. For example, nicotinic activity can produce intracellular Ca^{2+} signals that directly activate Ca^{2+} -dependent ion

channels or that initiate a cascade of enzyme activity. In addition to the well appreciated intracellular Ca^{2+} signals, there are activity-dependent decreases in extracellular Ca^{2+} throughout the central nervous system that provide important extracellular signals. Recently we have been investigating the dose-dependent direct modulation of neuronal (not muscle) nAChRs by extracellular Ca^{2+} .

Extracellular Ca^{2+} enhances currents through neuronal nAChRs and decreases currents through muscle nAChRs. The decrease in current through muscle nAChRs by extracellular Ca^{2+} is consistent with single-channel measurements that demonstrated a competition between Ca^{2+} and Na^+ or K^+ for occupancy of the permeation site(s) of the channel. When Ca^{2+} enters the permeation pathway, it moves through the channel more slowly than the monovalent cations. Therefore, Ca^{2+} decreases the macroscopic currents through the muscle nAChR channels by decreasing the underlying single-channel conductance. In contrast, currents through neuronal nAChRs are enhanced by extracellular Ca^{2+} even though the single-channel conductance decreases. Several results, including the lack of a voltage-dependence for modulation, indicate that the effect arises from a direct action of external Ca^{2+} on neuronal nAChRs not requiring a Ca^{2+} influx into the cell. In addition, the modulation can be a mechanism for Ca^{2+} -induced synaptic plasticity because rapid changes in external Ca^{2+} modulate cholinergic spontaneous synaptic currents in superior cervical sympathetic neurons. Experiments with ganglionic nAChRs from sympathetic neurons and chromaffin cells indicated that Ca^{2+} directly alters the probability of postsynaptic nAChRs being open. Inhibition of cholinergic currents by chlorisondamine, which blocks only open channels and becomes trapped in the pore, showed that the modulation is not by a mechanism that activates a previously unresponsive population of nAChRs. Taken together the results indicate that activity-dependent decreases in external Ca^{2+} , which occur throughout the nervous system, could directly underlie a rapid negative-feedback mechanism that decreases the postsynaptic response at cholinergic synapses. When external Ca^{2+} is decreased, presynaptic Ca^{2+} currents and transmitter release also are diminished. Thus, these presynaptic and postsynaptic mechanisms could combine to potently and rapidly depress highly active cholinergic synapses until the external Ca^{2+} concentration recovers.

Overall the research shows that nAChRs can act both as initiators of Ca^{2+} signals and as targets for Ca^{2+} -dependent modulation.

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Pertain references of our research include the following:

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*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

NOTES

MONDAY, NOVEMBER 7, 1994

(AFTERNOON, SECOND SESSION)

SYNAPTIC MODULATION AND PHOSPHORYLATION

Chair: Dr. S. Heinemann; Co-Chair: Dr. Y. Aracava

- | | | |
|-------|----------------|---|
| 15:30 | Silinsky, E.M. | What is the Cause of Neuromuscular Depression at Physiological Levels of Acetylcholine Release? |
| 16:00 | Huganir, R. | Regulation of Neurotransmitter Receptors by Protein Phosphorylation |
| 16:30 | Role, L.W. | Long-Term Regulation of Nicotinic Acetylcholine Receptor Expression and Ion Channel Function in Neurons |
| 17:00 | Barnard, E.A. | The Receptor Families of Ion Channels Gated by Extracellular Transmitters, from Nematode to Man |
| 17:30 | — | Refreshments |

WHAT IS THE CAUSE OF NEUROMUSCULAR DEPRESSION AT PHYSIOLOGICAL LEVELS OF ACETYLCHOLINE RELEASE?

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HISTORICAL OVERVIEW

Repetitive activation of the skeletal neuromuscular junction produces neuromuscular depression, a phenomenon that occurs as a consequence of a progressive reduction in the number of acetylcholine (ACh) quanta released by a nerve impulse (Katz, 1966). Depression is most dramatically manifested in patients with certain neuromuscular disorders such as myasthenia gravis; in this neuromuscular disease depression can be severely debilitating. Most attempts to quantify neuromuscular depression have been based on the assumption that this phenomenon is due to a reduction in the available vesicular store of ACh (Martin, 1966). While depletion of vesicular stores of ACh contributes to depression at enormous, unphysiological ACh outputs, there is no evidence to support the depletion hypothesis at normal levels of ACh release.

In the 1970's, it was found that ATP was released in stoichiometric amounts together with the neurotransmitter ACh from motor nerve endings (Silinsky, 1975). Based upon the evidence that ATP and its hydrolysis products inhibited the release of ACh, it was suggested that ATP released by motor nerve impulses could indeed be the mediator of neuromuscular depression,

In the 1980's, it was found that exogenous ATP needs to be hydrolyzed to adenosine and it is the action of adenosine on specific adenosine receptors that inhibits ACh release. This led to the modified model in which adenosine derived from the hydrolysis of endogenously-released ATP is the mediator of neuromuscular depression. This putative purinergic model of neuromuscular depression is presented in Fig. 1 below. For the convenience of the listener, the sites numbered

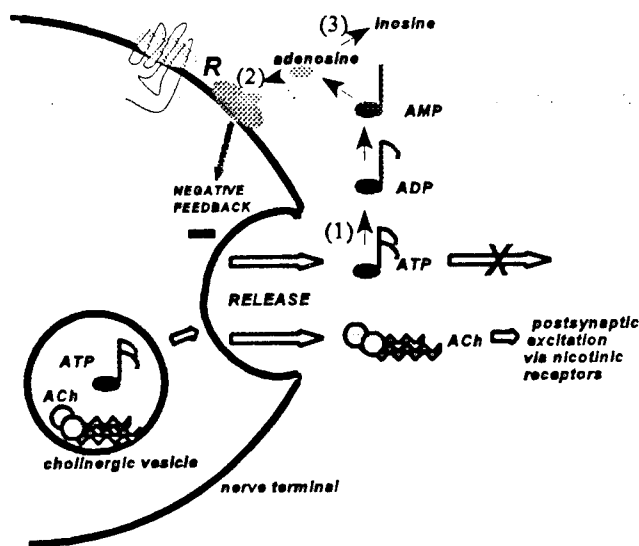


Fig. 1

(1)-(3) illustrate the loci of action of several highly-selective reagents that were used to evaluate the importance of negative feedback by purines in the process of neuromuscular depression as follows: site (1)- α, β -methylene ADP; site (2)-adenosine receptor antagonists; site (3)-adenosine deaminase.

In the 1990's, we tested the model shown in Fig. 1 in more detail and very recently found it to be an accurate depiction of the mechanism of neuromuscular depression (Redman and Silinsky, 1994). A description of these recent studies follows.

METHODS

Physiologically-functional ACh secretion was measured using intracellular recording of end-plate potentials (EPPs) from the innervated region of skeletal muscle (the end-plate region). Normal Ringer solution contained 1.8 mM Ca^{2+} and sufficient concentrations of tubocurarine or α -bungarotoxin to reduce EPPs below threshold for the generation of muscle action potentials. Reagents used in this study for their actions on purines were largely devoid of effects on postjunctional sensitivity to ACh; hence changes in the size of the EPP in the presence of drugs that alter the actions and concentrations of adenosine or during neuromuscular depression reflect changes in the magnitude of ACh release. In some experiments, we made simultaneous measurements of perineural Ca^{2+} currents and EPPs in 'Ca²⁺ current Ringer'. 'Ca²⁺ current Ringer' contained modest concentrations of the K⁺ channel blockers 3,4,-diaminopyridine (DAP, 100 μM) and tetraethylammonium (TEA, 250 μM). Such K⁺ channel blockade reduced the K⁺ current to allow sufficiently accurate measurements of the Ca^{2+} component of the perineural current without causing the profound depletion of ACh stores that would prevent reliable measurements of EPPs. Ca²⁺ current Ringer also contained 0.9 mM Ca^{2+} , 10 mM Mg^{2+} , 115 mM NaCl, 2 mM KCl and either tubocurarine or α -bungarotoxin.

In previous studies on the action of endogenous adenosine, considerable leakage of endogenous adenosine occurred, leakage that was unlikely to be associated with synaptic function. To minimize this basal purine efflux and hence to evaluate better the importance of endogenous adenosine in neuromuscular depression, we did the following: i) A thin preparation, the cutaneous pectoris nerve-muscle preparation of the frog was employed. This muscle is only a few cell layers thick in the region of examination; thus the leaching of endogenous adenosine to the surface of the muscle as a consequence of deep fiber anoxia in thick neuromuscular preparations was reduced or eliminated. ii) Dissections were performed carefully to minimize cut muscle. iii) Nerve stimulation prior to experimentation was avoided. iv) Local rapid superfusion was used to apply adenosine receptor reagents. This produced a high local synaptic concentration of these reagents and did so with a rapid onset of action (<50msec). Finally, v) Highly selective adenosine antagonists, specifically 8-cyclopentyl 1,3,dipropylxanthine (DPCPX) and 8-cyclopentyltheophylline (CPT), were used. .

EXOGENOUS ATP MUST BE HYDROLYZED TO ADENOSINE TO INHIBIT ACh RELEASE

At normal levels of ACh release and low frequencies of nerve stimulation (e.g., 1 every 10 sec), ACh release is well maintained. Under these stimulation conditions, α, β -methylene ADP (50 μM), an agent that inhibits ecto 5'-nucleotidases and therefore prevents the degradation of ATP to adenosine (see site (1), Fig. 1), prevented the inhibitory effects of ATP on ACh release. Likewise, the highly selective A₁ adenosine receptor antagonist (DPCPX-see site (2), Fig. 1)

prevented the inhibitory effect of ATP on ACh release. These data suggest that exogenous ATP needs to be hydrolyzed to adenosine to inhibit ACh release (much as is depicted for endogenous ATP in Fig. 1).

ENDOGENOUS ADENOSINE DERIVED FROM ATP IS THE MEDIATOR OF NEUROMUSCULAR DEPRESSION.

In contrast to the effects at low frequencies of stimulation (1 stimulus per 10 or 20 sec), when the frequency of nerve stimulation is increased to 0.5 to 1 Hz and greater, prejunctional depression of ACh ensues. We used three types of exogenous agents to evaluate the model of Fig. 1. First, we used α, β -methylene ADP, which blocks at site (1) in Fig. 1, i.e. blocks the degradation of ATP to the active purine adenosine. If the model is correct, then this agent should block neuromuscular depression. This prediction was borne out both at the frog and mammalian neuromuscular junction (mouse phrenic nerve-hemidiaphragm). With respect to sites (2) and (3) in Fig. 1, if endogenous adenosine is the mediator of neuromuscular depression, then selective adenosine receptor antagonists (which act at site (2), R=receptor) should eliminate neuromuscular depression. Furthermore, neuromuscular depression should be eliminated by the addition of adenosine deaminase to the bathing fluid. This enzyme acts at site (3) to degrade adenosine to inosine, which is inactive on adenosine receptors. Again, these predictions were borne out; the selective adenosine receptor antagonists DPCPX and CPT as well as exogenous adenosine deaminase, fully reversed neuromuscular depression. In addition, during maximal neuromuscular depression, the inhibitory effect of exogenous adenosine derivatives is blocked by occlusion from the endogenously released adenosine.

The remaining experiments were made on frog muscle in Ca^{2+} current Ringer, which contained modest concentrations of K^+ channel blockers. This solution, in which the level of ACh release was elevated approximately 2.5 fold that of normal solutions, allowed us to examine inter-impulse regulation of evoked ACh release by endogenous adenosine and to make simultaneous measurements of Ca^{2+} currents and evoked ACh release. It was found that neuromuscular depression and the reversal of depression by adenosine receptor antagonists or adenosine deaminase occur without effects on the Ca^{2+} components of the perineural currents measured simultaneously. Exogenous adenosine receptor agonists also have no effect on membrane ionic currents in frog motor nerve ending. In contrast, either a reduction in the extracellular Ca^{2+} or the addition of N-type Ca^{2+} channel blockers such as ω -conotoxin, Co^{2+} , Cd^{2+} or aminoglycoside antibiotics (streptomycin, neomycin or gentamicin) produced a parallel fall in EPP amplitude and Ca^{2+} currents.

DETECTION OF QUANTAL ATP RELEASE FROM MOTOR NERVE ENDINGS

There is published evidence that over a prolonged time course (minutes), the release of adenosine derivatives from nerve endings occurs from stimulated rat and frog motor nerve. To provide the final support for the purinergic model of neuromuscular depression shown in Fig. 1, it is necessary to demonstrate that quantal ATP release occurs over a msec time scale in conjunction with quantal ACh release. To perform such experiments, we made use of the discovery that acutely dissociated celiac neurons from the guinea-pig possess both nicotinic ACh receptors and highly sensitive P_2 receptors for ATP, with both nicotinic and purinergic receptors linked to a cation channel (Silinsky and Gerzanich, 1993). These excised ATP-gated cation channels have a

chord conductance of approximately 22 pS (at a holding potential of -50 mV). Experiments performed in 1990 and 1991, showed that ATP is indeed the mediator of fast excitatory synaptic transmission between cultured coeliac neurons (Silinsky et al., 1992), a result subsequently reproduced by others. In this original discovery, we used excised outside-out patches containing ATP-gated ion channels to sniff the extracellular milieu for the evoked release of ATP in coeliac neuronal cultures. Conditions have been described to detect the release of ACh from frog motor nerve endings using outside-out patches containing ACh receptors (Grinnell et al., 1989). Evidence for the concomitant quantal release of ATP and ACh from frog motor nerve using this patch sniff method will be described in this presentation.

SYNCHRONOUS EVOKED ACH RELEASE IN THE ABSENCE OF CALCIUM ENTRY IS ANTAGONIZED BY ADENOSINE

We have found that multiquantal EPPs may be generated by nerve impulses in the absence of calcium ion entry provided alkaline earth cation activators of secretion are provided by lipid vesicles (liposomes)-see poster by Watanabe et al. in this meeting. Such release is antagonized by adenosine receptor agonists, providing further evidence that the target site for the inhibitory effects of adenosine is beyond the site of calcium ion entry.

SUMMARY AND CLINICAL IMPLICATIONS

These results suggest that under the conditions of these experiments, adenosine derived from endogenously-released ATP is the exclusive mediator of neuromuscular depression. This inhibitory effect of adenosine in frog occurs by a mechanism unrelated to an action on membrane ionic channels; rather, it appears that adenosine is capable of reducing the ability of Ca^{2+} to promote the secretory process. The precise target site for the action of adenosine at the secretory apparatus is unknown but speculations will be provided in the presentation.

Regardless of the precise nature of the inhibitory mechanism, these results could have important clinical relevance. Specifically, if a similar mechanism of neuromuscular depression occurs in human skeletal muscle, then a selective prejunctional A_1 adenosine antagonist suitably targeted to motor nerve endings could be used to treat the debilitating neuromuscular fatigue that occurs when myasthenia gravis patients attempt to sustain repetitive neuromuscular activity.

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REGULATION OF NEUROTRANSMITTER RECEPTORS BY PROTEIN PHOSPHORYLATION.

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The precise regulation of synaptic efficacy in the mammalian central nervous system is fundamental for learning, memory, motor control, and sensory processing, as well as synaptogenesis. Currently, the molecular mechanisms underlying synaptic plasticity involved in these crucial processes are topics of intense investigation. Neurotransmitter receptors mediate signal transduction at the postsynaptic membrane of synaptic connections between cells in both the central and peripheral nervous systems. This pivotal role in the mechanism of synaptic transmission suggests that neurotransmitter receptors may be potential targets at which synaptic plasticity could occur. Modulation of the function, expression, or density of neurotransmitter receptors in the postsynaptic membrane could have profound effects on the efficacy of synaptic transmission. A variety of evidence has suggested that protein phosphorylation of neurotransmitter receptors is a common mechanism for the regulation of receptor function (1). Over the past several years, we have used the nicotinic acetylcholine receptor (AChR) as a model system to study the role of protein phosphorylation in the regulation of neurotransmitter receptor function. In addition, we have recently begun to study the role of protein phosphorylation in the regulation of the major excitatory and inhibitory neurotransmitter receptors in the CNS, the glutamate receptors and GABA_A receptors.

Nicotinic Acetylcholine Receptor Phosphorylation

The AChR mediates synaptic transmission at the postsynaptic membrane of the neuromuscular junction. The AChR is a pentameric complex of four types of subunits (α , β , γ and δ) in the stoichiometry of $\alpha_2\beta\gamma\delta$. Each subunit has been proposed to have a large N-terminal extracellular domain, for hydrophobic transmembrane α -helices (M1-M4) with a major intracellular loop between M3 and M4. The AChR is multiply phosphorylated *in vitro* and *in vivo* by cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and a protein tyrosine kinase (PTK). The phosphorylation of the AChR subunits occurs on the major intracellular loop of each subunit. Phosphorylation of the receptor by each of these protein kinases appears to regulate the rate of desensitization of the receptor. The phosphorylation of the AChR in muscle is under the control of a variety of nerve derived factors including acetylcholine itself and the neuropeptide calcitonin gene-related peptide. Tyrosine phosphorylation of the AChR is regulated *in vivo* and *in vitro* by neuronal innervation of muscle. Experiments to characterize the components of nerve involved in the regulation of tyrosine phosphorylation have demonstrated that agrin, a neuronal extracellular matrix protein, regulates tyrosine phosphorylation in a manner similar to innervation. Agrin is thought to be released from the neuron and to mediate the nerve-induced clustering of the AChR at the neuromuscular junction (2). Recent studies have shown protein tyrosine kinase inhibitors block agrin-induced tyrosine phosphorylation of the AChR and agrin-induced clustering of the AChR. These results suggest that tyrosine phosphorylation of the AChR may be involved in the regulation of clustering of the AChR at synapses by agrin and neuronal innervation.

In recent studies we have examined the role of a synaptic peripheral membrane 43K protein in AChR clustering. The 43K protein has long been thought to play a role in AChR clustering at synapses because it is found at equimolar concentrations with the AChR at synapses and extraction of the 43K protein increases the lateral mobility of the AChR in the synaptic membrane (3). However, the strongest evidence supporting a role of the 43K protein in AChR clustering comes from experiments where the 43K protein was co-expressed with the AChR in heterologous cells. Expression of the AChR in *Xenopus* oocytes or quail QT-6 fibroblasts leads to the diffuse distribution of the AChR on the cell surface (3). In contrast, co-expression of the 43K protein and the AChR results in the aggregation of the AChR on the cell surface. To investigate whether

tyrosine phosphorylation may be involved in 43K protein-induced clustering of the AChR we examined 43K protein-induced receptor clusters and demonstrated that they contain high levels of phosphotyrosine as detected using immunofluorescent labeling with anti-phosphotyrosine antibodies. Co-expression of the 43K protein with the AChR resulted in the induction of tyrosine phosphorylation of the β and δ subunits of the receptor. Site-specific mutagenesis of the tyrosine phosphorylation sites on the β and δ subunits of the receptor eliminated the tyrosine phosphorylation of these subunits. However, the mutations did not block clustering of the receptor, nor did they eliminate the high levels of phosphotyrosine that colocalized with the 43K protein-induced receptor clusters. In addition, transfection of 43K protein alone dramatically increased tyrosine phosphorylation of several cellular membrane proteins. These results support the role of 43K protein in neuron- and agrin-induced clustering of the nicotinic receptor and suggest that tyrosine phosphorylation of membrane proteins but not the nicotinic receptor itself, is important for receptor clustering.

Glutamate Receptor Phosphorylation

Glutamate receptors are the major excitatory neurotransmitter receptors in the central nervous system and play major roles in synaptic transmission, synaptic plasticity, synaptogenesis, and excitotoxicity (4,5). Glutamate receptors can be divided into three major pharmacological subclasses referred to as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors based on their preferred agonists. Molecular cloning studies in several laboratories have led to the isolation of cDNAs encoding many different glutamate receptor subunits. To date, fourteen different glutamate receptor subunits have been isolated which belong to the AMPA (GluR1-4, A-D), kainate (GluR5-7, KA1-2) and NMDA (NR1, 2A-D) receptors (4,5). Like the other ligand-gated ion channels, such as the nicotinic acetylcholine receptor and the GABA_A receptor, glutamate receptors have been proposed to consist of pentameric complexes of these homologous subunits. In addition, these subunits were originally proposed to have a similar transmembrane topology as other ligand gated ion channels with a large extracellular N-terminal domain, four transmembrane domains (M1-M4) and a major intracellular loop between M3 and M4.

Modulation of ionotropic glutamate receptor function is a central mechanism underlying plasticity at excitatory synapses and, in some cases, may be mediated by the direct phosphorylation of these receptors (6). For example, long-term potentiation (LTP) in hippocampal CA1 pyramidal cells is the persistent increase in excitatory synaptic transmission that occurs after brief periods of high frequency stimulation. A variety of studies have suggested that phosphorylation of glutamate receptors may play a role in the increased synaptic efficacy observed during LTP. In addition, long-term depression in cerebellar Purkinje cells is a persistent decrease in synaptic efficacy at excitatory synapses after coactivation of the two excitatory inputs to these cells. Several studies have suggested that this synaptic depression may be due to phosphorylation of glutamate receptors.

To investigate the regulation of glutamate receptors by protein phosphorylation we have used molecular, biochemical and physiological techniques to directly examine the phosphorylation and modulation of recombinant and neuronal glutamate receptors (6). Expression of the GluR6 subunit in human embryonic kidney (HEK) cells produces functional kainate receptors that can be studied biochemically and electrophysiologically. Using this heterologous expression system we have shown that the GluR6 subunit is directly phosphorylated by cAMP-dependent protein kinase (PKA). Moreover, intracellular application of PKA increases the peak amplitude of the glutamate response measured using patch clamp techniques. Site-specific mutagenesis of a single serine residue (S684) within the major intracellular loop between M3 and M4 completely eliminated the phosphorylation of this site and blocked the potentiation of the glutamate response by PKA.

Expression of the GluR1 and GluR2 subunits in heterologous expression systems produce functional receptors which have many of the characteristics of AMPA receptors in neurons. In recent studies, we have shown that the GluR1 subunit expressed in HEK cells is basally phosphorylated by an unidentified protein kinase and can be phosphorylated by PKA and a protein tyrosine kinase. We are currently attempting to identify the location of these phosphorylation sites and characterize the functional effects of these phosphorylations.

In addition we have examined the phosphorylation of the NMDA receptor NR1 subunit in transfected HEK cells and in neurons in culture. Protein kinase C phosphorylates the NR1 subunit on several distinct sites. Using site-specific mutagenesis, we have found that most of these phosphorylation sites are located on a single alternatively spliced exon, which had previously been proposed on the extracellular side of the membrane. We have recently found that the NR1 subunit is also phosphorylated by PKA on distinct serine residues within the same C-terminal region phosphorylated by PKC. These results suggest that alternative splicing of the NR1 mRNA regulates its phosphorylation by PKC and PKA and that mRNA splicing is a novel mechanism for regulating the sensitivity of glutamate receptors to protein phosphorylation. This data also suggests that the proposed transmembrane topology model for glutamate receptors is incorrect. Assuming that the transmembrane topology of AMPA, kainate and NMDA receptor subunits are similar, our results on GluR6 and NR1 phosphorylation have suggested that there are either no membrane crossings, or an even number of transmembrane domains between the PKA site phosphorylated in GluR6 (S684) and the PKC sites phosphorylated on the NR1 subunit (S889, S890, S897). It is interesting to note that the intracellular location of the C-terminus is actually consistent with our previous immunocytochemical data using C-terminal antibodies.

Recent studies have demonstrated that the NR2A and NR2B subunits of the NMDA receptor are phosphorylated by PKC and by a protein tyrosine kinase. Current studies are addressing the functional role of these phosphorylations on NMDA receptor function.

Summary

Our results demonstrate that neurotransmitter receptors are directly modulated by protein phosphorylation and that protein phosphorylation is a complex mechanism for the regulation of the function of these receptors. Moreover, these studies suggest that phosphorylation of neurotransmitter receptors may play a central role in the regulation of synaptic transmission and in higher brain function such as learning and memory.

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Long term regulation of nicotinic acetylcholine receptor expression and ion channel function in neurons.

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Current knowledge of the mechanisms controlling transmitter-receptor expression in neurons is still rather sparse. Although it is clear that the number of transmitter-gated channels available for activation on neurons are subject to both short term regulation (or modulation) as well as long term control, the cellular and molecular machinery underlying both phenomena in neurons are just emerging. Thus, previous work in numerous laboratories have documented that the number, subtypes and cellular distribution of ligand-gated channels are precisely controlled during early neuronal development and synapse formation. In addition, synaptic receptors on neurons can be modulated so that the number of activatable receptors as well as the rate of activation and inactivation can be altered. These modulatory effects may contribute to short term synaptic plasticity.

My laboratory is interested in how both short and long term changes in transmitter-gated channels are implemented in CNS and PNS neurons. We have focused our studies on the developmental regulation and modulation of neuronal nicotinic acetylcholine receptors (nAChRs) with the hope that at least some of the processes underlying nAChR regulation in neurons would be shared with those of its muscle nAChR (m-nAChR) cousins. To date our studies indicate both common and divergent mechanisms in the regulation of neuronal and muscle-type AChRs as well as in the modulation of these receptors. This abstract summarizes our studies of the molecular events in long term regulation of nAChR ion channel expression, function and distribution in specific classes of CNS and PNS neurons.

Early embryonic nAChR profile Like m-AChRs, nAChRs are potently regulated during neuronal development and synaptogenesis. Our studies of embryonic lumbar sympathetic ganglion neurons (LSG) as well as neurons of the medial habenula nucleus (MHN) reveal that the levels of receptor expression are low and that the nAChR channels are diffusely distributed over the soma, at early stages of development (1,2). In these studies we examined the properties of nAChR channels in MHN and LSG neurons as well as the levels of nAChR subunit gene expression in LSG. Neurons were assayed within hours of extirpation as well as after maintained incubation (3-7 days) in primary culture. Both assays suggest that the profile of nAChRs expressed is unchanged in neurons maintained *in vitro* in the absence of either presynaptic input or target cells. Thus, under our *in vitro* conditions, both classes of neurons retain the transmitter and nAChR profiles expressed at the time of their removal *in vivo*. Important aspects of this phenotypic stability with maintenance *in vitro* are the virtual absence of non-neuronal cells as well as the absence of synaptic interactions between the dispersed LSG or MHN neurons. Both these classes of early embryonic CNS and PNS neurons express multiple nAChR channel subtypes, distinguished by their conductance (γ), open time kinetics and the concentration of agonist required for long term inactivation (or desensitization i.e. interburst intervals ≥ 60 secs; 1,2). Although each subtype is evident in most patches assayed, recordings from both MHN and LSG neurons at early stages of development reveal the predominance of small γ (≤ 35 pS), short burst duration nAChR channels. These smaller γ events are the most sensitive to agonist-induced desensitization in that they enter prolonged

closed states with exposure to $\geq 2 \mu\text{M}$ agonist, whereas the interburst intervals of larger γ nAChR subtypes are ≤ 15 seconds with 5 fold higher agonist concentration.

Late embryonic nAChR profile Examination of the properties of nAChR channels as well as the levels and cellular distribution of the nAChRs at later stages of embryogenesis reveals an array of changes in nAChRs expressed by MHN and LSG neurons (1,2). In these studies, neurons were removed at a time when synaptic projections to and from the MHN and LSG were well underway (E17-20). Examination of the amplitude of agonist activated macroscopic currents in E17-20 LSG neurons reveals 4-10 fold increases in the peak of the inward current (reference 1 and unpublished data). Parallel assay of the nAChR subunit gene profile reveals 2-10 fold increases in the expression of the $\alpha 3, \alpha 5, \alpha 7$ and $\beta 4$ nAChR in E17 LSG (3). Both late stage MHN and LSG neurons express multiple nAChR subtypes, but the predominant channel subtypes are higher γ , long burst duration channels with decreased apparent affinity for agonist and slow inactivation with continued exposure to $\geq 10 \mu\text{M}$ nicotine. Finally, late stage MHN and LSG neurons express a novel nAChR subtype (≈ 70 pS) and, in striking contrast to early stages, repeat patch clamp recording at multiple sites on individual neuronal soma reveal that nAChRs segregate *by channel subtype*, into high density single-class patches.

Do changes in nAChR subunit composition underlie the developmental changes in nAChR channel profile? We have begun to test the notion that the concomitant changes in nAChR subunit gene expression and the alterations in nAChR channel profile that occur during development are causally related. Again, our thinking is an extension of studies of the m-AChR where developmental alterations in the profile of m-nAChR subunits underlie the concomitant changes in m-nAChR channels (Sakmann and colleagues). Our approach has involved two complementary techniques: selective subunit deletion by antisense oligonucleotides and heterologous expression of selective subunits. In both paradigms macroscopic and single channel currents are assayed to examine the contribution of specific subunits to nAChR channel properties. To date we have examined the role of $\alpha 2, \alpha 3, \alpha 4$, and $\alpha 5$ in both MHN and LSG neurons using the subunit deletion and/or subunit combination approaches (we have deleted $\alpha 7$ as well, but so far only from LSG). In summary, these studies, combined with the perspective provided by biochemical and molecular studies of Berg and Lindstrom and their colleagues as well as work in our laboratory, suggest that early on the principle ligand binding subunit in LSG nAChRs is $\alpha 3$, whereas $\alpha 2$, and to a lesser extent, $\alpha 4$ are the principle players in early stage MHN nAChRs. Our recent findings further suggest that the developmental decrease in the apparent agonist affinity and concomitant increase in the number of large γ nAChRs are due to the inclusion of $\alpha 5$ in the nAChR complexes (Ramirez-Latorre, Yu, and Role, unpublished), a particularly intriguing result in view of previous attempts that failed to demonstrate any functional role of $\alpha 5$.

Presynaptic input vs. target innervation as determinants of nAChR expression Currently, we are directing our studies to test the role of presynaptic input vs. target contact in regulating nAChR subunits and channel subtypes during embryonic development. LSG neurons are removed prior to input or target contact *in vivo* and placed *in vitro* with presynaptic visceral motoneurons (VMN), which readily and rapidly establish synaptic innervation. Assay of the nAChR currents in neurons innervated *in vitro* (in the absence of target cells) reveals that input alone recapitulates some, but not all, of the changes in nAChRs seen with *in vivo* development. Assay of the levels of nAChR subunit genes in these innervated LSG neurons by quantitative PCR, also indicates that presynaptic input is similar, but not equivalent, to *in vivo*

development in regulating nAChR expression. Key differences include the increase in $\alpha 3$ expression, which is greater with innervation alone and the increases in both $\alpha 5$ and $\alpha 7$, which are more substantial during development or with target innervation alone (see below).

Examination of "naive" LSG neurons maintained *in vitro* with target cells (but without presynaptic input) suggests that target innervation may alter both nAChR subunit and channel profiles in a manner that is complementary to the changes induced by presynaptic input alone. Thus, innervation of target cardiocytes decreases the number of lower γ subtypes and upregulates the expression of nAChRs ≥ 70 pS. Furthermore, quantitative PCR assay of the levels of nAChR subunit genes in neurons that have innervated target reveals parallel decreases in the levels of $\alpha 3$ and $\beta 4$ expression with concomitant increases in $\alpha 5$ and $\alpha 7$ expression. Thus input and target may collaborate to change the nAChR profile from primarily small γ , $\alpha 3\beta 4$ nAChRs to high γ , $\alpha 3\alpha 5\beta 4$ channels.

Candidate signaling molecules for the regulation of neuronal nAChR expression.

The above studies and previous work in my laboratory have also revealed that aspects of the input- and target-induced changes in the levels of neuronal nAChR expression are mimicked by treating LSG neurons with medium conditioned by VMN or target cells. These findings indicate an important role of soluble signaling molecules in regulating neuronal nAChR expression and are reminiscent of extensive studies by Fischbach and colleagues demonstrating that muscle AChRs are regulated by soluble motoneuron-derived factors including the recently cloned and characterized ARIA (see symposium abstract, this volume). In view of these parallels as well as the common embryological origins of visceral and somatic motoneurons, we searched for ARIA-related molecules that might be expressed in VMN and specifically regulate neuronal nAChR expression. We have now cloned and expressed a novel splice variant [which we have named "nARIA"; details presented at this meeting, (5)] with a unique N terminal domain that includes an ≈ 120 bp cysteine-rich sequence and lacks the characteristic Ig domain. To date our findings are consistent with nARIA mediating the effects of VMN neurons in the regulation of neuronal nAChRs, *in vivo* (5). Furthermore, whereas nARIA increases both nAChR currents and nAChR subunit gene expression in LSG neurons similarly to presynaptic input, ARIA decreases nAChR channel and subunit expression in these neurons, in a manner reminiscent of LSG-target cardiac myocyte innervation.

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THE RECEPTOR FAMILIES OF ION CHANNELS GATED BY EXTRACELLULAR TRANSMITTERS, FROM NEMATODE TO MAN

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The transmitter gated ion channels, constituting one of the 3 general structural Classes of the membrane-bound signal transducing receptors, are divided into 2 sub-Classes, the first where the transmitter acts extracellularly and the second (employing completely different superfamilies of subunits to the first) where the signalling molecule (e.g. IP₃, cyclic GMP, etc.) arrives intracellularly (Barnard, 1992). All the subunits in this Class have structures that can assemble in an oligomer (usually heteromeric, sometimes apparently homomeric) surrounding a membrane pore. Transduction is via the opening of a cation or an anion channel [or, for one member recently cloned (the ATP-gated K⁺ channel), the closing of a cation channel], activated by the binding of the signalling ligand. The extra-cellularly activated transmitter-gated ion channels perform fast signalling, since their transduction is independent of any intracellular or membrane-diffusible factor. Eight receptor types are known in this sub-Class (Table 1), all of which have had subunit structures revealed by DNA cloning. It is of interest that at least 3 unrelated superfamilies are employed.

On these eight, the most detailed structural and functional information has been available for the nicotinic acetylcholine (ACh) receptor of the muscle/electric organ type, as described fully in previous sections of this Symposium. In this case it has been definitively established that all of the subunits span the membrane to form a pentamer which encloses a central ion channel (Unwin, 1993). The latter form has also been established recently by electron optical analysis of the GABA_A receptors (Nayeem et al, 1994) and similarly for the 5-HT₃ receptor (R. Beroukhim, F.G. Boess and I.L. Martin, unpublished). There is other evidence for a pentameric composition for the neuronal nicotinic ACh receptors and for the glycine receptor (as noted elsewhere in this Symposium). These 5 receptor types named in this paragraph form one superfamily by their sequence homologies (low but definite) and their identical distributions along the subunit sequence of 4 hydrophobic (deduced transmembrane, TM) domains.

The glutamate receptors show either no sequence homology, or occasional vestigial traces of possible homology, to the other cationic channels in this receptor Class. Their distribution of hydrophobic domains along the subunits is different, and variable; the N-terminal domains are much larger than in the latter receptors and sometimes also the C-terminal domains. The number, membrane topology and secondary structure of their true membrane-spanning segments is at present still an open question. For these reasons they are regarded as forming a different superfamily (Table 1), but since this still is closer to the aforementioned 5 receptors than to any other known receptors, it is assigned as Class 1b, as compared to Class 1a of those five.

The GABA_A and glutamate receptors have in common with the neuronal nicotinic ACh receptors construction of the receptor from a repertoire of subunit types, on a combinatorial plan. This is illustrated here for the GABA_A receptors, now known to be encoded by at least 17 genes in the vertebrates. These produce five different subunit types (with 30-40% identity between pairs)

(α , β , γ , δ and ρ), each except δ having known multiple isoforms (65-80% identity): 6 α -subunits ($\alpha 1 - \alpha 6$), 4 β -subunits ($\beta 1 - \beta 4$), 4 γ -subunits ($\gamma - \gamma 4$), a δ -subunit, as well as $\rho 1$ and $\rho 2$ in the retina. (For references, see Barnard, 1992). In addition, at least the $\gamma 2$ mRNA exists in two differently-distributed alternatively-spliced variants. The pentamers of the GABA receptors are constructed, therefore, by combination from among these 18 subtypes of the subunits. Thus, for the majority of native GABA_A receptors, which recognise benzodiazepines, α , β and γ subtypes should co-occur in one receptor (Pritchett et al, 1989). Some other cases however, are now known to occur. Present evidence from both direct and indirect approaches suggests a theoretical maximum of the order of 500 permitted subunit combinations in the pentamers, and that while the actual number in the nervous system must be far below this there is nevertheless a great multiplicity. One transmitter type, GABA, can thus be employed in a great variety of neural circuits by virtue of the distribution of many receptor types for it; these are assembled from a repertoire of at least 18 subunit forms in a combinatorial system, 5 at a time.

How do these combinations differ in their function? To answer this, we must note that, on the GABA receptor molecule, experimentally 9 binding sites have been found so far for different ligand structures (the GABA site, the benzodiazepine site, the convulsant site, the neurosteroid site, etc). These determine either the binding of the transmitter or the channel structure or the modulation (for positive or negative gain) of those activities. At least some of them should correspond to sites for natural modulatory messengers. The occurrence and behaviour of these 9 sites varies, of course, with the protein structure, so that high functional diversity is created by the combinatorial design. The same is presumed to apply to combinatorially - generated sets of pentamers for the neuronal nicotinic ACh receptors, the non-NMDA receptors and the NMDA receptors. Little or no multiplicity has been found, in contrast, in the muscle nicotinic or the 5-HT₃ or the glycine receptors.

Starting from the background of great diversity within 4 of the receptor types in this Class, recent findings will be reported on (i) evolutionary changes in their sequences, down to those of simple nervous systems, which throw light on the minimum constant requirements, and (ii) a very different type of glutamate receptor subunit, which can produce a new type of diversity in the glutamate receptors, with a form which is intermediate between the NMDA and non-NMDA receptors.

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Table 1. Transmitter-gated ion channels

Ligand	Ion Selectivity*	Super-family	Trans-membrane domains
<i>Extra-cellularly activated</i>			
GABA _A	Cl ⁻ , HCO ₃ ⁻	IA	4
Glycine	Cl ⁻ , HCO ₃ ⁻	IA	4
ACh (nicotinic, muscle type)	Na ⁺ , K ⁺ , (Ca ²⁺)	IA	4
ACh (nicotinic, neuronal)	Na ⁺ , K ⁺ , Ca ²⁺	IA	4
5-HT ₃	Na ⁺ , K ⁺	IA	4
Glutamate: non-NMDA	Na ⁺ , K ⁺ , (Ca ²⁺)	IB	?
Glutamate: NMDA	Na ⁺ , K ⁺ , Ca ²⁺	IB**	?
ATP (P _{2X})	Ca ²⁺ , Na ⁺ , Mg ²⁺	II***	3

* Parentheses denote: (1) for the muscle nicotinic receptor, the relative Ca²⁺ permeability is only one-sixth that for the neuronal ACh receptor (Mulle, C. et al., Neuron 8, 135-143, 1992); (2) for non-NMDA receptors, there may be Ca²⁺ permeability, depending on, e.g., whether GluR-2 or certain forms of GluR-6 are present (see the papers from the groups of S. Heinemann and of P. Seeburg here).

** One report (Smirnova, T., Stinnakre, J. and Mallet, J., Science 262, 430-433, 1993) has proposed that another, presynaptic NMDA receptor type exists, which would be in a superfamily of its own, with only one TM domain.

*** A Surprenant and co-workers (Valera, S. et al., Nature, 1994 in press) have recently cloned and expressed a cDNA encoding a rat P_{2X} receptor. This is active as a homo-oligomer, formed by identical subunits deduced to have 2 hydrophobic TMs plus a β-loop P region such as lines the channel in voltage-gated K⁺ channels.

*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



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MONDAY, NOVEMBER 7, 1994

18:15-19:45

THE AARON I. GROLLMAN MEMORIAL LECTURE

PROF. P. GREENGARD

Pre- and Post-synaptic Modulation of Synaptic Transmission

Interactions of signal transduction pathways in the brain

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We have been studying the integration of signal transduction pathways in a variety of neuronal tissues. One particularly attractive model system has been the medium spiny neurons of the neostriatum. A key molecule involved in the integration of various signals impinging upon the medium spiny neurons is a protein called DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein, apparent $M_r=32,000$). We have found twelve signal transduction pathways which are involved in regulation of the state of phosphorylation of DARPP-32. When DARPP-32 is phosphorylated on threonine 34, it is converted from an inactive substance to a potent inhibitor of protein phosphatase-1, one of the major protein phosphatases in the brain. A major substrate for protein phosphatase-1 is the electrogenic ion pump Na^+ , K^+ -ATPase. Na^+ , K^+ -ATPase is active in its dephosphorylated form and less active when it is phosphorylated at serine 943, so that the state of phosphorylation of Na^+ , K^+ -ATPase controls membrane potential and neuronal excitability. A variety of first messengers, second messengers, protein kinases and protein phosphatases are involved in regulating the state of phosphorylation of DARPP-32. As a result, the DARPP-32/protein phosphatase-1/ Na^+ , K^+ -ATPase cascade integrates signal inputs from a variety of nerve cells, leading to a coordinated regulation of neuronal excitability.

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TUESDAY, NOVEMBER 8, 1994

(MORNING, FIRST SESSION)

NICOTINIC ACETYLCHOLINE RECEPTOR

Chair: Dr. D. Drachman; Co-Chair: Dr. D. Pumplin

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|-------|-------------|---|
| 8:00 | Cohen, J.B. | Membrane Spanning Regions of the Nicotinic Acetylcholine Receptor: Structure of the Protein-Lipid Interface and the Ion Channel |
| 8:30 | Karlin, A. | Structures Involved in Binding, Gating, and Conduction in Acetylcholine Receptors |
| 9:00 | Hucho, F. | Investigations of the Secondary Structure of the Nicotinic Acetylcholine Receptor |
| 9:30 | Unwin, N. | Three-Dimensional Structure of the Acetylcholine Receptor in the Closed and Open States |
| 10:00 | -- | Coffee Break -- Poster Session |

MEMBRANE SPANNING REGIONS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR: STRUCTURE OF THE PROTEIN-LIPID INTERFACE AND THE ION CHANNEL

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To describe the mechanism of permeability control by nicotinic acetylcholine receptors (AChRs), we have been using protein chemistry techniques to define structural domains of the membrane-bound AChR isolated from Torpedo electric organ. Radiolabeled probes with selectivity based upon either topologic specificity or binding site affinity are incorporated covalently into the AChR, and then labeled subunits are isolated so that sites of labeling can be identified by N-terminal sequence analysis of labeled peptides generated by proteolysis and isolated by SDS-PAGE and HPLC.

To identify the structure of the AChR at its lipid interface, we have characterized the sites of incorporation of a series of uncharged, hydrophobic, photoactivatable probes. Molecules of this class partition into the lipid bilayer, and they may also potentially bind to hydrophobic pockets within the protein interior. Protein labeling from the lipid interface can be distinguished from labeling from an interior binding pocket, since incorporation of a radiolabeled probe from the lipid interface would be nonspecific, i.e. independent of the concentration of nonradioactive probe, while labeling from a binding pocket would be inhibitable by excess, non-radioactive probe. For each probe the character of the photogenerated intermediate will determine its relative reactivity with nearby nucleophiles (SH, OH) as opposed to aliphatic side chains. By examining a series of compounds with different structures and excited states, we hope to establish a map of the AChR at the lipid interface that is insensitive to the particular side chains at the interface.

The probe we have characterized most extensively is [¹²⁵I]TID, first introduced by Brunner and Semenza (1). TID is a precursor to a reactive, short-lived carbene that reacts with aliphatic sidechains, albeit at lower rate than with nucleophilic side chains. In initial experiments (2) we established that TID acts both as a nonspecific probe of the AChR-lipid interface and also, to our surprise, as a potent nicotinic noncompetitive antagonist that binds with high affinity ($K_d \sim 4 \mu\text{M}$) within the AChR ion channel. The amino acids contributing to the [¹²⁵I]TID binding site were determined (3) in two different states of the AChR: the resting state (in the absence of agonist) and the desensitized state that is stabilized at equilibrium in the presence of agonist. In the resting state the specific photolabeling of the AChR was restricted to the M2 hydrophobic segments of each subunit, and within M2 only β -subunit Leu-257 and Val-261 as well as homologous residues in the M2 regions of each other subunit were labeled. This pattern of labeling provided the first localization of the site of binding of a closed channel blocker and also led to the proposal that even in the absence of TID the permeability barrier in the closed channel of the AChR was a

plug made up of these aliphatic side chains from positions 9 and/or 13 of the M2 segments from each subunit.

In contrast to the specific photoincorporation of [^{125}I]TID into M2 segments of each subunit, its incorporation into M3 and M4 segments of each subunit was the same both in the presence and absence of agonist or in the presence of excess nonradioactive TID, consistent with nonspecific photoincorporation from the lipid interface (4). [^{125}I]TID reacted with five residues in α -M4 that would be positioned on the common face of an α -helix distributed over 5 helical turns. For M4 of β -, γ -, and δ -subunits, [^{125}I]TID reacted with only two or three residues, a number insufficient to make a clear, independent assignment of secondary structure. Analysis of the sites of [^{125}I]TID labeling in M3 segments provided extremely strong evidence that these hydrophobic segments must also have α -helical secondary structure. In δ -M3 labeled amino acids include Met-293, Ser-297, Gln-301, Val-304 and Asn-305, as well as Ile-288 preceding M3. Residues at three or four of the corresponding positions were labeled in β -M3 and γ -M3, respectively. The distribution of these labeled residues define a strip in contact with lipid that extends over three (β - and γ -subunits) or four (δ -subunit) helical turns.

Unlike the similar patterns of labeling within each of the M3 and M4 segments that are both independent of either the concentration of nonradioactive TID or the presence of agonist, the pattern of photolabeling of each M1 segment appears unique. For α -M1 labeling was insensitive to agonist or nonradioactive TID, with labeled residues (Cys-222, Leu-223, Phe-227, and Leu-228) in a region of primary structure following the proline (Pro-221) that is common to M1 segments of all ligand-gated ion channels. β -M1 was not photolabeled at all, while labeling of δ -M1 occurred in the presence, but not in the absence, of agonist at residues preceding (Phe-232, Ile-233) and following (Cys-236) Pro-235 (3). Unfortunately, γ -M1 has not yet been isolated in sufficient purity to identify the sites of photolabeling, sites that we would predict to be similar to those seen in δ -M1. The nonequivalence of the labeling patterns of the M1 segments suggests that there are agonist-induced changes in structure in δ -M1 (and we predict γ -M1) that are likely to be coupled to the agonist-dependent changes in structure of the M2 domain that were revealed by the pattern of specific photolabeling of M2 segments by [^{125}I]TID.

To extend the map of the AChR-lipid interface, we are using as a second probe [^3H]diazofluorene ([^3H]DAF), another carbene precursor developed to characterize the lipid interface of membrane proteins(5). While TID reacts essentially via an excited state singlet carbene, DAF generates a carbene with substantial triplet state character that would favor C-H bond insertion. While many of the same residues in the M3 and M4 segments are labeled by both compounds, [^3H]DAF also reacts with residues not labeled by TID, including Phe-443 in β -M4 and Trp-453 in γ -M4 as well as Arg-282, Lys-285, and Lys-290 in the β -, γ -, and δ -M3 segments, respectively. Overall the distribution and periodicity of the labeled residues in the M3 and M4 segments further strengthens the conclusion that these segments are organized as transmembrane α -helices with a similarly defined face of each helix in contact with

lipid.

A second area of study concerns the structure of the ion channel and the mechanism of action of nicotinic noncompetitive antagonists. Molecules that bind within the ion channel will act as noncompetitive antagonists, and photoaffinity analogs of such drugs serve as powerful probes of the structure of the ion channel in the different AChR conformational states. This was seen particularly clearly with [¹²⁵I]TID that reacts with amino acids in different regions of the M2 channel domain in the resting and desensitized states of the AChR (3). However, a wide range of drugs act as noncompetitive antagonists, including aromatic amines (phencyclidine), aliphatic alcohols, peptides (substance P), steroids and detergents. It is likely that only some of these compounds bind within the ion channel, while others interact with other regions of the AChR crucial for the propagation of a change in structure from the agonist site to the channel. Alternatively, some of the compounds may, in fact, act from the AChR-lipid interface.

To further define the structure of the closed channel, we have determined the sites of photoincorporation of [³H]tetracaine ([³H]TET, dimethylaminoethyl-p-butylaminobenzoate), which is positively charged at physiological pH. Tetracaine is an unusual aromatic amine noncompetitive antagonist because it binds with high affinity ($K_d=0.3 \mu\text{M}$) to the AChR in the resting state and only weakly to the AChR in its desensitized state ($K_d=30 \mu\text{M}$). Nevertheless, [³H]TET binds to one site per AChR monomer, and it binds in a formally competitive manner with drugs such as phencyclidine that bind preferentially to the AChR desensitized state. Upon UV irradiation (>300 nm), [³H]TET is specifically photoincorporated with similar, low efficiency (~0.1%) into each AChR subunit. Specific photolabeling is restricted to the M2 segments of each subunit, and the labeled residues include each of the aliphatic amino acids labeled by [¹²⁵I]TID. However, for some subunits [³H]TET is photoincorporated into additional residues. Thus, in α -subunit Ile-247 is labeled as well as Leu-251 and Val-255, three residues that define a narrow strip extending over 3 turns of an α -helix. In δ -subunit Ala-268 is labeled as well as Leu-265 and Val-269. These results indicate that in the closed state of the AChR the channel domain has sufficient structural flexibility to allow [³H]TET to reach the level of α Ile-247.

In contrast to tetracaine with its selectivity for the AChR in the resting, closed channel state, the undecapeptide substance P acts as a desensitizing noncompetitive antagonist that binds with micromolar affinity to the desensitized AChR and at least 100-fold more weakly to the AChR resting state(6). Substance P appeared to bind to a site in the AChR distinct from the site of binding of [³H]phencyclidine. To identify the regions of the AChR interacting with substance P, we made use of a novel photoreactive derivative originally developed (7) for studies of the NK-1 receptor, the high affinity receptor for substance P that is a member of the G-protein-coupled receptor superfamily. The photoreactive analog contained p-benzoyl-l-phenylalanine (BPA) in place of Phe⁸ and an amino terminal tyrosine for radioiodination ([¹²⁵I]BPA⁸-SP). Because the BPA residue and the ¹²⁵I are in separate residues of the peptide probe, N-terminal protein sequence analysis can not

be used to determine the exact amino acid residue(s) of the AChR that are photocrosslinked to the substance P probe. In the absence of agonist, [¹²⁵I]BPA⁸-SP (1nM) was photoincorporated nonspecifically into each AChR subunit, while the presence of agonist resulted in a 2-fold increase in δ-subunit photolabeling that was inhibited by excess substance P or by meproadifen, an aromatic amine noncompetitive antagonist. The sites of specific photoincorporation in δ-subunit were mapped by use of *S.aureus* V8 protease to a 14 kDa fragment (δV8-14) extending from Ile-192 to Glu-280, while further fragmentation of δV8-14 with trypsin and V8 protease established that the sites of specific photoincorporation were restricted to δSer-253 to Glu-280, a region containing only the M2 hydrophobic segment. Thus in the presence of agonist the photoreactive benzoylphenylalanine at position 8 of substance P must have access to at least the N-terminal (extracellular) end of the M2 region.

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STRUCTURES INVOLVED IN BINDING, GATING, AND CONDUCTION IN
ACETYLCHOLINE RECEPTORS

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Important clues about the mechanisms of ACh-binding, gating, and cation-conduction by the ACh receptors can be gained from the assignment of functional roles to particular amino-acid residues in the sequences of the subunits. Such assignments have been made either by the identification of the sites of functionally significant covalent modifications of the receptor, or by the determination of the functional consequences of site-directed mutagenesis, or by a combination of these approaches.

To identify channel-lining residues, we have substituted cysteine for individual residues in the membrane-spanning segments of the receptor and have determined the accessibility of these substituted cysteines to highly polar, charged sulfhydryl-specific reagents (Akabas et al., 1992 and submitted). We call this approach the substituted-cysteine-accessibility method (SCAM). The sulfhydryl-specific reagents are charged derivatives of methanethiosulfonate (MTS), namely MTSEthylammonium (MTSEA), MTSEthyltrimethylammonium (MTSET), and MTSEthylsulfonate (MTSES) (Stauffer and Karlin, 1994). These derivatives are about 0.6 nm in diameter and 1 nm long. The positively charged MTSEA and MTSET are both conducted by the open receptor channel and thus have access to the entire channel lining. We assume that these highly polar MTS derivatives react only at the water-accessible surface of the receptor and that within the membrane-spanning domain of the receptor the only water-accessible surface is the lining of the channel.

We infer the accessibility of the substituted cysteines to the MTS derivatives from the irreversible effects of these reagents on the ACh-induced current in oocytes expressing the mutant receptors. Thus, this approach works only with cysteine-substitution mutants that are functionally expressed. It is the accessibility of a residue, not the effects of its mutation, that is sought by this approach. Also, if the function of the mutant is nearly wild-type then the structure of the mutant is also likely to be nearly wild-type, and the cysteine side chain is likely to be positioned relative to the channel lumen similarly to the wild-type side chain that it replaced.

We have applied SCAM to the M2-membrane-spanning segment of mouse-muscle-receptor alpha subunit (Figure 1). One at a time, 22 consecutive residues, from E241 to E262, were mutated to cysteine and the mutant alpha subunits, together with wild-type beta, gamma, and delta subunits, were expressed in *Xenopus* oocytes. Twenty-one of the twenty-two mutants gave robust currents in response to ACh. Only the mutant K242C failed to express.

With the the receptor in the closed state, MTSEA, added

extracellularly, irreversibly inhibited channel function in ten of the cysteine-substitution mutants (Figure 1). Of these ten residues, seven residues, either the same residue in alpha or the aligned residue in another subunit, were previously implicated by others as contributing to channel structure or function (reviewed in Karlin, 1993). Our results unambiguously identified all ten residues in alpha as contributors to the channel lining; furthermore, the newly identified L250 and L258 have implications for the secondary structure of M2.

Figure 1. Mouse alpha M2 segment.

(x) Cysteines substituted for these residues were accessible to MTSEA added extracellularly to the closed channel; (+/-) accessibility was increased/decreased in the presence of ACh. (*) Prior evidence for location in channel. in=intracellular; ex=extracellular.

	in						ex															
	241		251				261															
	E	K	M	T	L	S	I	S	V	L	L	S	L	T	V	F	L	L	V	I	V	E
	X	XX	X	XXX	X	X	X	X														
	+	-		-+	+	+	-															
	*	*	*	**	*		*															

The patterns of accessibility of the residues from E241 to S248 and from L253 to E262 are compatible with an alpha-helical conformation. The accessibilities of L250, L251, and S252, however, are not compatible with an alpha-helix, and these residues are more likely to be in a beta-strand or other extended conformation. Thus, we conclude that M2 forms a broken helix, interrupted by a short extended link.

Assuming that the receptor is nearly five-fold symmetrical around the channel, then the cytoplasmic end of the channel is likely to be lined by five alpha-helices from the cytoplasmic end of M2, one from each subunit. At the wider extracellular end of the channel, the wall is formed by five alpha-helical M2 segments alternating with five other segments. Previous photolabeling experiments (DiPaola et al., 1990) and preliminary results with SCAM indicate that these other segments are M1; i.e., that M1 also contributes to the channel lining at its extracellular end.

The accessibility of residues in the closed state of the channel sets limits on the location of the gate. That E241C was accessible to MTSEA in the closed state implies that the gate is at least as close to the cytoplasmic end of the channel as is E241. It follows that the gate is formed either by the loop between M1 and M2 or by the loop between M3 and M4.

In the presence of ACh, the accessibility of some residues changed. These changes were not due to the opening of the gate per se, because some residues became more accessible and some became less accessible, and these changes were distributed over the length of M2 (Figure 1). Furthermore, the accessibility of T244 and S248 was not changed by ACh. Therefore, channel opening is associated with changes in the structure of the segments lining the channel over its entire length.

MTSET reacted with only a subset of the residues accessible to MTSEA. These differences are attributable to the larger size and rigidity of MTSET and the fact that it not only reacts covalently with cysteines, it is also a reversible, partial agonist of the receptor. MTSES, which is negatively charged,

reacted with E262C and with V255C. This latter result is surprising and implies that the channel becomes cation-selective at a position more cytoplasmic than V255. It is clear that the charged residues, including E262, at the extracellular end of the channel are not responsible for the cation selectivity of the channel.

It is the binding of ACh to the two ACh binding in the extracellular domain of the receptor that drives the opening of the gate, which is in the cytoplasmic domain. The locations of the ACh binding sites in the interfaces between the alpha and gamma (or epsilon) subunits and between the alpha and delta subunits are consistent with ACh binding inducing a relative movement of the subunits; this could be the mechanism whereby the conformational change initiated at the binding sites is transmitted across the membrane (Czajkowski et al., 1993). By crosslinking and peptide mapping, we identified delta D180 as a residue that is 0.9 nm from the binding site residues, alpha C192 and C193 (Czajkowski and Karlin, 1991 and in preparation). Furthermore, the mutant delta D180N bound ACh two orders of magnitude less strongly than wild type. Mutation of the homologous residue in gamma, D174 to N similarly decreased the affinity for ACh by two orders of magnitude (Martin et al., in preparation). Thus, these negatively charged residues in gamma and delta contribute to the binding of ACh, most likely interacting electrostatically with the positively charged quaternary ammonium group of ACh (Stauffer and Karlin, 1994).

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INVESTIGATIONS OF THE SECONDARY STRUCTURE OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

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The nicotinic acetylcholine receptor (AChR) is the prototype of the four transmembrane helix (4TM) receptors (1, 2). The ligand-gated ion channels GABA_A-R, Gly-R, AChR (nicotinic), the ionotropic glutamate receptors and the 5HT₃-R belong to this class (3). Their transmembrane folding patterns are predicted with the help of computer algorithms based on hydropathy plots (4-7). Relatively little experimental evidence supports these predictions. On the contrary, for the AChR it has been questioned recently whether the transmembrane domain is made up exclusively of helices. In an electron microscopical investigation (8) it was shown that at 9 Å resolution per receptor subunit only one structure could be detected which had the dimensions of an alpha-helical rod. Located near the center of the protein, this helix seems to be the transmembrane sequence M2, shown to be part of the receptor's ion channel (9 - 11). Various evidence had indicated that M2 is indeed a helix. Site-directed mutagenesis experiments using the open channel-blocker QX-222 as a probe provided the first evidence for the helical nature of the channel-blocker binding site (12). Similarly, photoaffinity labelling experiments using the non-competitive inhibitor ³H-chlorpromazine were in favor of a helical conformation (13). Experimental data on the secondary structure are also available for M4. The periodicity of photolabelling with ¹²⁵I-TID (3-trifluoromethyl-3-(m¹²⁵I-iodophenyl)diazirine) indicated that M4 is exposed to the lipid environment and contains alpha-helical secondary structure. Analogous results were obtained for M3 (14).

We addressed the problem of the secondary structure within the transmembrane domain of the AChR using FTIR spectroscopy (15). We treated an AChR-rich membrane preparation with proteinase K to remove the extramembrane part of the receptor. The remaining protein (about 25% of the total receptor protein) accounts approximately for the four hydrophobic transmembrane stretches. FTIR spectra obtained with this preparation gave two sets of important data: the secondary structure composition of

the transmembrane domain could be calculated, and, by measuring the linear dichroism, the orientation of secondary structure elements with respect to the membrane plane could be investigated. It turned out that the portion of the receptor which is not accessible to the proteinase (presumably because it is buried within the lipid bilayer) contained about equal amounts of alpha-helical and beta-structure (fig. 1). Furthermore, the alpha-helices seem to span the membrane with a uniaxial orientation, while no such orientation could be observed for the beta-structures (fig. 2). This lack of unidirectional orientation corresponds to the 'diffuse' beta-structures deduced from electron microscopical investigations. One could imagine that the beta-structures function as scaffolds for the pore-forming alpha-helices.

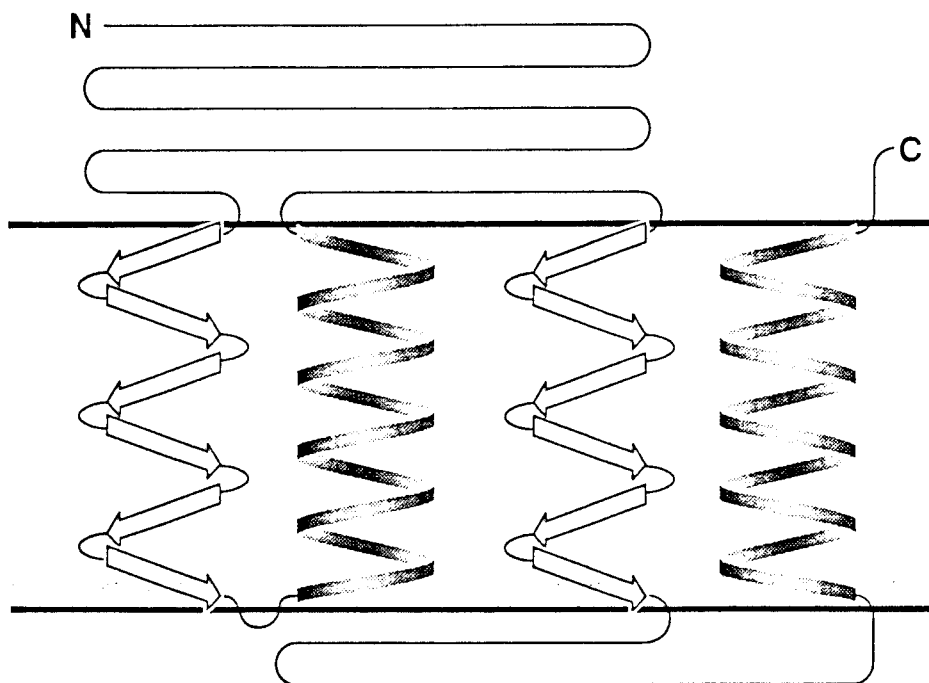


Fig. 1 - Schematic representation of the transmembrane folding of AChR subunits. The model takes into account that there are about equal amounts of α - and β -structure and that M2 and probably M4 are α -helices. The β -structures (arrows), assigned to M1 and M3, are drawn pointing in different directions. FTIR measurements (linear dichroism) have shown that the intramembrane β -structures are not oriented uniaxially. The intramembrane peptides have not yet been identified, therefore the number of membrane passings in this model are hypothetical. Good experimental evidence exists for both the N- and the C-terminus being on the extracellular side of the membrane.) (Figure reproduced from Hucho et al., TIBS, in press.)

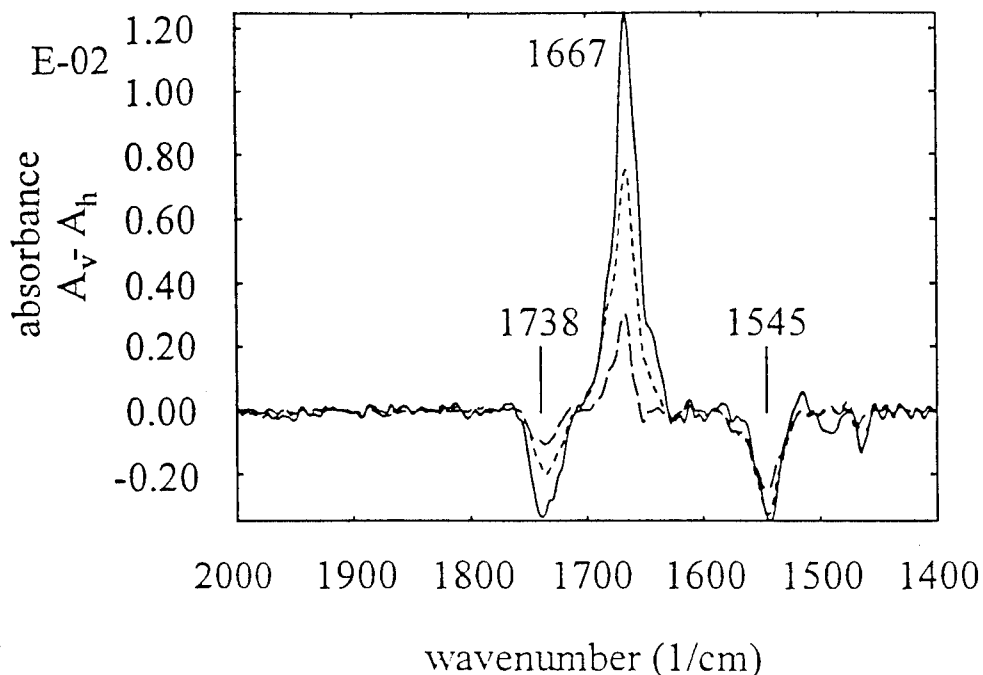


Fig. 2 - Linear dichroism of an oriented sample of receptor-rich membranes after proteolysis and removal of the cleavage products at various tilt angles with respect to the incident probe beam (taken from Ref. 15). Shown is the difference of the absorbance of vertically polarized (A_v) and horizontally polarized (A_h) IR light. Sample tilt: 19.2° dashed line; 25.6° dotted line; 32.0° solid line. The positive Amide I difference band at 1666 cm^{-1} and the negative Amide II difference band at 1545 cm^{-1} can be assigned to bands of oriented α -helical structures (Rothschild and Clark, 1979).

Of course these conclusions are based on two assumptions which still need experimental proof: first, it is assumed that the secondary structure is not altered by the proteinase K treatment; second, the membrane preparation used for FTIR spectroscopy contains exclusively amino acid sequences immersed in the lipid bilayer. While it is difficult to provide experimental support for the first assumption, the second assumption can be experimentally investigated. Presently we are attempting to isolate and identify the protein sequences present in the membrane preparation used for spectroscopy. Purification of these lipophilic and very similar peptides using HPLC poses considerable problems. We therefore turn to matrix-assisted laser desorption ionization (MALDI) mass spectrometry. After partial delipidation of the intramembrane protein, the transmembrane peptides resulting from proteinase K treatment become accessible to analysis by this technique. As a first (and still preliminary) result, we detected a peptide corresponding to α Y278 through α S304. This sequence comprises M3, extending on the C-terminal side into the sequence

previously thought to represent the extra-membrane loop connecting M3 and M4.

Although it is premature to propose a realistic model of the transmembrane domain of the AChR, our conclusion from these experiments is the following: the transmembrane domain of the AChR most likely contains considerable amounts of beta-structure. The hydrophobic sequences M1 and M3 probably form beta strands to a large extent, whereas M2 and M4 may be composed largely of alpha-helices. The alpha-helical structure seems to be uniaxially oriented, while the beta strands are not.

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Three-dimensional structure of the acetylcholine receptor in the closed and open states.

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This talk will describe the three-dimensional structure of the nicotinic acetylcholine receptor in the closed- and open-channel forms, at 9Å resolution. The two conformations have been determined by electron crystallographic analyses of tubular crystals of *Torpedo* post-synaptic membranes embedded in amorphous ice.

At low resolution,¹ the receptor is seen to consist of a ring of five similar subunits, 120Å long, which delineate through the membrane a central pathway for the ions. The pathway is narrow across the membrane, but widens into a cylinder which is 20-25Å in diameter and extends ~60Å into the synaptic cleft and 15-20Å into the interior of the cell.

By averaging the data obtained from >50,000 molecules it is possible to extend the resolution to 9Å, and hence visualize some elements of secondary structure within the five protein subunits.² In the extracellular domain of each subunit of the closed channel, about 30Å above the membrane surface, there is a group of three rods that are oriented predominantly perpendicular to the plane of the membrane and twist around each other as in a left-handed coil. These rods presumably are α -helices. Two of them line the entrance to the channel, and the third is on the outside. The distinctive appearance of the α subunits in this region suggests that the rods may be involved in forming the binding pocket for acetylcholine. Fluorescent energy transfer measurements also indicate that the binding pockets are 30-40Å above the membrane surface.³

In the membrane-spanning part of each subunit there is only one rod clearly visible, which forms the wall lining the pore, and so is assumed to be the transmembrane helix, M2. This rod does not form a straight path through the lipid bilayer, but bends, or kinks, near its mid-point, where it is closest to the axis of the pore, and tilts radially outwards on either side. It is flanked on the lipid-facing sides by a continuous rim of density, which seems likely to be composed of β -sheet.

A tentative alignment can be made between the three-dimensional densities and the sequence of M2, based on correlation of the appearance of the rods with a special pattern of amino acid residues in the sequence. This alignment places the charged groups at the ends of M2

symmetrically on either side of the bilayer, and a highly conserved leucine residue (Leu251 of the α subunit) at the level of the kink. It is suggested that leucine side-chains projecting into the pore from the kinks associate and create a tight hydrophobic ring, which closes the channel by making a barrier that hydrated ions cannot cross.

The structure of the open-channel conformation of the receptor was determined in the same way as for the closed channel, but from images of the *Torpedo* membranes activated by brief (<5ms) mixing with acetylcholine spray droplets before rapid freezing to trap the structural response. It can be demonstrated that this structure is distinct from that of the desensitized state, which involves a more substantial conformational change.

Comparison of this new conformation with the closed-channel conformation reveals that acetylcholine induces a twisting of the rods composing the binding pocket in the α_δ subunit, an action that seems to be coordinated with smaller changes in the α_γ subunit and the single subunit in between the two α subunits. These localized disturbances initiated at the level of the binding sites in the α subunits induce small rotations of the subunits extending the membrane-spanning pore.

The M2 helices surrounding the open pore have changed their configuration, but the rim lying outside them maintains its original shape, as if to act as a framework shielding the moving elements from the surrounding lipids. The lower portion of the open pore is lined by a right-handed barrel of α -helices, which constricts ion flow maximally near the intracellular membrane face. The concerted rotations of the subunits, triggered by acetylcholine, appear to underlie the mechanism by which the effect of its binding is communicated to the M2 helices to open the gate.

These experiments, to image the acetylcholine receptor first in the closed and now in the open channel form, have led to a better understanding of how the receptor works. The confinement of major disturbances to the main functional regions, the development of concerted rotational motions to disrupt the gate, and the transient formation of an α -helical barrel around the open pore are new principles revealed. Together, they provide a structural basis for explaining the remarkable effectiveness of this protein at the neuromuscular junction.

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*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

TUESDAY, NOVEMBER 8, 1994

(MORNING, SECOND SESSION)

NICOTINIC ACETYLCHOLINE RECEPTOR

Chair: Dr. J. Daly; Co-Chair: Dr. M. Eldefrawi

- | | | |
|-------|-----------------|--|
| 10:30 | Hall, Z. | Agrin and the Assembly of the Neuromuscular Junction |
| 11:00 | Heinemann, S. | Alpha 9: A New Acetylcholine Receptor with Novel Pharmacological Properties |
| 11:30 | Changeux, J.-P. | Functional Organisation and Dynamics of the Nicotinic Receptors: Recent Developments |
| 12:00 | -- | Lunch Break |

AGRIN AND THE ASSEMBLY OF THE NEUROMUSCULAR JUNCTION

Sugiyama, J., Ferns, M., Bowen, D. and Z. Hall

Department of Physiology, University of California, San Francisco, CA 94143

Formation of the specialized postsynaptic membrane at the neuromuscular junction depends on the exchange of signalling molecules by pre- and postsynaptic cells. One of these, agrin, is a component of the synaptic basal lamina which is synthesized and secreted by motor neurons, and is thought to cause acetylcholine receptors (AChRs) and other postsynaptic components to become concentrated at the endplate. (McMahan, 1990; Reist et al, 1992).

In collaboration with the laboratory of Richard Scheller, we have investigated the action of rat agrin on C2 mouse muscle cells and on genetic variants of C2 cells defective in the synthesis of proteoglycans that fail to form spontaneous AChR clusters (Gordon et al, 1993). Our experiments show that different agrin forms, generated by alternative splicing, have differences in biological activity (Ferns et al, 1992, 1993). The most active agrin form, which has inserts of 4 and 8 amino acids at the x and y splice sites, respectively, is found exclusively in the nervous system, whereas a less active form, without inserts at these two sites, is the predominant form in muscle (Hoch et al, 1993). When tested on the proteoglycan-deficient C2 variants, all forms of agrin were less active than on the parental C2 cells, suggesting that proteoglycans play a role in mediating the action of agrin in clustering AChRs.

We have recently used soluble, C-terminal fragments of the neural and muscle agrin forms to investigate the binding of agrin by muscle cells and by Torpedo electric organ (Sugiyama et al, 1994). Agrin bound well to a single component of muscle cell membranes and of Torpedo membranes with apparent molecular weights of 130-150 kD and 200 kD, respectively. Binding of agrin to each was calcium-dependent and was inhibited by heparin and dextran sulfate. Interestingly, both the soluble form of neural agrin, which is biologically active at a concentration of approximately 5pM, and the soluble form of muscle agrin, which is inactive at 5 nM, bound with approximately nM affinity.

Investigation with specific antibodies (generously provided by Kevin Campbell) demonstrated that the binding component in both muscle and Torpedo was alpha-dystroglycan, one of the complex of proteins that is associated with dystrophin in muscle cells (Ervasti et al, 1990). A monoclonal antibody to the carbohydrate portion of dystroglycan that inhibits the binding of laminin to the protein (Ervasti and Campbell, 1993) also was found to inhibit the binding of agrin.

The properties of dystroglycan in the proteoglycan-defective C2 variants that responded poorly to agrin was investigated using antibodies to the protein and carbohydrate portions of the molecule. Although dystroglycan was present in both variants, its electrophoretic mobility was altered in each case. Moreover, dystroglycan in both variants bound agrin less well than the dystroglycan from C2 cells.

Immunocytochemical staining of AChR clusters induced by agrin showed that dystroglycan was associated with the clusters. The induction of AChR clusters by agrin, however, was not inhibited by a monoclonal antibody that blocks agrin binding to dystroglycan.

What is the physiological role of the binding of agrin to dystroglycan? Our results are consistent with at least four possibilities: 1) dystroglycan could be the signaling receptor for agrin that is responsible for the initiation of AChR clusters; 2) dystroglycan could be an auxiliary protein for such a signaling receptor; 3) the interaction of agrin with dystroglycan could be involved in the growth and maintenance of AChR clusters, rather than in their initiation; 4) the binding of agrin to dystroglycan could mediate an action of agrin that is unrelated to AChR clustering. These possibilities and experiments related to them will be discussed.

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ALPHA 9: A NEW ACETYLCHOLINE RECEPTOR WITH NOVEL
PHARMACOLOGICAL PROPERTIES

Elgoyen, A.B., Johnson, D.S., Boulter, J., Vetter, D.E., and Heinemann, S.

Molecular Neurobiology Laboratory; The Salk Institute P.O. Box 85800 San Diego,
California 92138

We have identified , cloned, and functionally characterized a new member of the nicotinic receptor gene family, alpha 9. The alpha 9 subunit forms homomeric receptors which are functional and gated by acetylcholine. The alpha 9 receptor has a mixed pharmacology with features of both nicotinic and muscarinic receptor pharmacology. The primary structure of alpha 9 is related of previously characterized nicotinic receptor ligand gated channels. The alpha 9 gene has an intron-exon organization which is different from previously identified nicotinic receptor genes and may represent a new subfamily of nicotinic receptor genes.

The expression pattern of alpha 9 gene is unique when compared to other nicotinic receptors. Preliminary data shows that alpha 9 is expressed in the pars tubercularis of the hypophysis, the nasal epithelium, the hair cells of the cochlea and the tongue.

The alpha 9 gene is expressed in the inner and outer hair cells of the cochlea. The unique pharmacological properties of the alpha 9 receptor match those described for the cholinergic receptors present in vertebrate hair cells of the cochlea. We speculate that the alpha 9 receptor mediates the cholinergic efferent innervation of the cochlear hair cells which modulates the sensitivity and turning of individual hair cells to auditory stimulus.

Elgoyhen, A.B., Johnson, D.S., Boulter, J., Vetter, D.E., and Heinemann, S.: Alpha9: An Acetylcholine Receptor with Novel Pharmacological Properties Expressed in Rat Cochlear Hair Cells. CELL, **in press**.

FUNCTIONAL ORGANISATION AND DYNAMICS OF THE NICOTINIC RECEPTORS : RECENT DEVELOPMENTS

M.R. Picciotto, C. Léna, Y. Lallemand*, M. Zoli, A. Bessis, N. Le Novère, P. Brulet* and J.P. Changeux.

Laboratory of Molecular Neurobiology and *Laboratory of Molecular Embryology, Institut Pasteur, Paris, France.

Transgenic mouse lines lacking the $\beta 2$ -subunit of the neuronal nicotinic receptor were generated using the technique of homologous recombination. Homozygous mutant mice do not express the $\beta 2$ -subunit, are viable and can reproduce. Using *in situ* hybridization, there is no evidence of replacement of the $\beta 2$ -subunit with another known nicotinic β -subunit, and RNA levels of the $\alpha 4$ -subunit, the subunit thought to form functional channels with the $\beta 2$ -subunit in the brain, are normal in mutant mice.

Patch clamp analysis of neurons in brain slices from wild type and mutant mouse brains show marked differences in their response to nicotine. Neurons from anterior thalamic nuclei of wild type mice, which express high levels of the $\beta 2$ -subunit and do not express other known β -subunits, respond to nicotine application with an inward current, while neurons from the same brain regions of $\beta 2$ -mutant mice show no response to nicotine.

Quantitative receptor autoradiography using ^3H nicotine (4nM) demonstrates that high affinity nicotine binding is reduced by approximately 50 % in all brain areas in heterozygotes, and completely abolished in homozygotes.

Behavioral analysis is in progress.

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NOTES

TUESDAY, NOVEMBER 8, 1994

(AFTERNOON, FIRST SESSION)

SYNAPTOGENESIS AND DEVELOPMENT I

Chair: Dr. Z. Hall; Co-Chair: Dr. D. Burt

- | | | |
|-------|-----------------|---|
| 13:00 | McMahan, U.J. | Composition and Function of the Matrix in the Synaptic Cleft |
| 13:30 | Salpeter, M. | Building a Stable Neuromuscular Junction |
| 14:00 | Fischbach, G.D. | A Role for Tyrosine Kinases in Synapse Formation |
| 14:30 | Merlie, J.P. | The Role of 43K Rapsyn in Synapse Formation and Stabilization |
| 15:00 | -- | Coffee Break -- Poster Session |

COMPOSITION AND FUNCTION OF THE MATRIX IN THE SYNAPTIC CLEFT

McMahan, U.J.

Department of Neurobiology Stanford University School of Medicine. Stanford, CA 94305.

Electron microscopists nearly 40 years ago recognized that the composition of the matrix in the synaptic cleft differs from that elsewhere in the nervous system (1,2,3). They noted, for example, that when nervous tissue was routinely stained with osmium tetroxide and other metals the synaptic cleft was far more opaque. It was only natural to wonder what role matrix composition might play in synaptic function but methodology at that time severely limited exploration of this problem. Over the last 15 years the extraordinary development of techniques for molecular biology has made it possible to examine in considerable detail components of the matrix in the synaptic cleft of the neuromuscular junction. At this synapse the matrix is organized into a basal lamina which is strongly adherent to both the pre- and postsynaptic plasma membranes and it is continuous with the basal lamina that coats the muscle fiber's extrajunctional plasma membrane. Basal lamina is deposited on the surface of many cell types throughout the body. It consists primarily of strongly associated collagenous and non-collagenous glycoproteins. The basal lamina in the synaptic cleft of the neuromuscular junction has constituents common to all basal laminae. However, it also contains several "synapse specific" proteins (4). Synapse specific functions for most of these are obscure, but one, acetylcholinesterase, has long been known to play a direct role in synaptic transmission by terminating the action of the neurotransmitter on the muscle fiber. There is also good evidence that another, agrin, mediates the motor neuron-induced formation of acetylcholine receptor aggregates in the postsynaptic membrane (5). The receptor aggregates are necessary for sufficient depolarization of the muscle fiber during synaptic transmission to generate an action potential. I will discuss the results of recent experiments aimed at determining how acetylcholinesterase and agrin are associated with basal lamina and what role this association plays in the function of these proteins.

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BUILDING A STABLE NEUROMUSCULAR JUNCTION

Salpeter, Miriam M.

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The vertebrate neuromuscular junction (nmj) is uniquely organized both morphologically and molecularly to produce a very stable structure with a high concentration of specific molecules. These include acetylcholine receptors (AChRs), acetylcholinesterases (AChEs) sodium channels and various other extracellular and cytoplasmic molecules. One of the remarkable aspects of the nmj is that although innervation is required for its initial development many junctional specializations can survive subsequent denervation. This is due to a variety of "memory molecules" which seem to be deposited in the vicinity of the nmj, and probably expedite accurate reinnervation. Yet not all junctional properties are irreversible after denervation. In my presentation I will deal with one such reversible property at the nmj, i.e. AChR degradation.

In innervated nmj's the AChR's are concentrated at the top third of the junctional folds at an amazingly high density of about 10,000 AChR molecules (or 20,000 ACh binding sites) per μm^2 of dense post-synaptic membrane. This receptor is very stable (degradation $t_{1/2} \sim 10$ d). While high density receptor aggregation at the nmj is initially induced in response to innervation, this is one feature that is retained after denervation. The stabilization of the AChR on the other hand is a reversible process which has a complex time course and regulation (see also abstracts by Rufeng Xu and Jim O'Malley).

When the nmj is denervated AChR degradation is affected in 2 ways: 1) The degradation rate of the stable AChRs that were synthesized and localized at the nmj before denervation (called Rs) accelerate to a $t_{1/2}$ of 3 days and 2) new rapidly degrading receptors (called Rr, $t_{1/2} \sim 1$ d) are synthesized and replace the Rs as they degrade. Many experiments in our laboratory aimed at determining how these two receptor types differ and how stabilization at the nmj is established have led to several already published results. These include: 1) that the accelerated Rs receptors can be restabilized in the post-synaptic membrane upon reinnervation (Salpeter et al., 1986; Andreose et al., 1993); the Rs can thus be modulated after insertion in the membrane and can have either an 8-10 day or a 3 day half life, dependent on the state of innervation. 2) Dibutyryl cyclic-AMP (DB-cAMP) can mimic the effect of reinnervation in stabilizing the Rs receptors in muscles studied in organ culture (Shyng et al. 1991). 3) Electrical stimulation initiated at the time of denervation can prevent the Rs acceleration. However, once Rs has accelerated to a $t_{1/2}$ of 3 days, electrical stimulation cannot reverse the process (Andreose et al., 1993). 4) After denervation, Rr receptors replace the Rs as they degrade, yet even in the denervated muscles $\sim 10\%$ of the receptor population remains in the form of the accelerated Rs with a $t_{1/2}$ of ~ 3 d (Shyng & Salpeter, 1990). 5) The Rr receptor is not stabilized to the adult half life by reinnervation (Shyng & Salpeter, 1990; Andreose et al., 1993), by dBcAMP (Shyng et al., 1991; O'Malley et al., 1993) or by direct electrical stimulation (Andreose et al., 1993). We conclude that the nerve cannot alter the degradation rate of Rr receptor and can only down regulate Rr expression (i.e. in the presence of the nerve or electrical stimulation, Rr is not expressed in the membrane). 6) In muscle cells grown in tissue culture 80-90% of the AChRs behave like the Rr in denervated muscle ($t_{1/2} \sim 1$ day) and 10-20% behave like accelerated Rs ($t_{1/2} \sim 3$ d) (O'Malley et al., 1993). The AChRs on muscle cells in culture thus provide a model system for studying regulation of muscle AChR.

Based on this background, I will report recent, as yet unpublished, experimental results. This will involve some information on the mode of action of dBcAMP, our current thinking on the structural differences between the Rs and Rr receptors, and the events occurring during the process of reinnervation of denervated muscle.

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A NEW CLASS OF NEUROTROPHIC FACTORS: HANG. Fischbach, G.D., Corfas, G., Falls, D., Goodearl, A., Loeb, J., Krauss, R., Rosen, K., Sandrock, A. Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA.

At developing neuromuscular junctions, AChRs begin to accumulate in the immediate vicinity of motor nerve terminals within a few hours after nerve - muscle contact is established. Motor nerves do not initiate AChR subunit gene expression. At chick junctions that form *in vitro*, the majority of receptors present at the earliest stages are newly synthesized molecules. AChR subunit mRNA and assembled AChRs (α bungarotoxin binding sites) can be detected in proliferating myogenic cells in the limb bud before the motor axons invade the muscle forming regions. However, motor nerves do increase the local rate of AChR synthesis and insertion in the immediate vicinity of developing synapses. In chick muscle, the increase amounts to 4 - 5 fold compared to nearby extrasynaptic regions.

We have purified a protein from chick brain that induces the synthesis of nicotinic ACh receptors in cultured chick myotubes. The protein, named ARIA for its acetylcholine receptor inducing activity, is a member of a family of proteins that are synthesized by one gene. Diversity is generated within the family by alternative mRNA splicing.

Our hypothesis is that one or more isoforms of ARIA are synthesized in motor neurons, that a fragment is released into the synaptic cleft, and that it binds to specific muscle membrane receptors to activate processes that lead to the accumulation of AChRs at developing neuromuscular junctions. Several experiments support this view. The ARIA gene is expressed in motor neurons at the time when nerve-muscle synapses first form (embryonic day 4 -5 in the chick and 14 - 15 in the rat). The protein is present in motor nerve terminals although with the antibodies presently available, the first staining is not detectable until about one week after neuromuscular junction formation. Either, the immunohistochemical assay is not sensitive enough to detect ARIA at early times, or an unrecognized isoform is present, or ARIA does not play a role in the initial accumulation of AChRs.

ARIA purified from brain tissue migrates in SDS containing gels as a broad band centered at about 40 kD. It is apparently synthesized as part of a larger precursor protein because a cDNA isolated from an embryonic chick brain library predicts a protein of 602 amino acids. The protein contains a stretch of hydrophobic amino acids near the middle of the sequence that, presumably, spans the lipid bilayer. The purified protein is probably derived from the extracellular half. The extracellular half of the molecule contains an EGF - like domain that includes six cysteines, a short "spacer" region and an immunoglobulin - like region.

ARIA is homologous to the recently cloned Neu Differentiation Factors (NDFs), the heregulins, and to Glial Growth Factor (GGF). NDFs and heregulins were purified based on their ability to stimulate the phosphorylation of tyrosine residues in a 180 - 185 kD transmembrane receptor. GGF was purified on the basis of its ability to stimulate the proliferation of Schwann cells. ARIA does stimulate tyrosine phosphorylation of a 185 kD protein in skeletal muscle that is likely to be a member of the HER (or neu or erbB) family of receptor tyrosine kinases, but exactly which one remains to be determined. It is probably not neu, thought originally to be the sole binding site for NDF (hence the name). Little of the tyrosine phosphorylated p185 in muscle cells can be immunoprecipitated by anti - neu antibodies under conditions that do precipitate nearly all of the neu protein.

Despite the diverse effects that led to the discovery of the heregulin NDF/GGF/ARIA

family, it appears that all of these proteins are encoded by one gene. Variation is due to alternate splicing. The degree of variation is enormous with new isoforms appearing with increasing frequency. It is comforting that a few simplifying rules have emerged. One of the most important is that the EGF-like domain is sufficient for AChR inducing activity. We have expressed this part of the molecule as a bacterial fusion protein and recovered full AChR inducing activity in the expected dose range.

Several important issues remain. First, what exactly is the role of ARIA and related proteins at the developing neuromuscular junction? In addition to the initial accumulation of receptors, ARIA may play a role in the maturation of the AChR phenotype and in the maintenance of the adult receptor number. About one week after birth, two weeks after the initial accumulation of junctional receptors, the γ gene is suppressed and the ϵ gene is expressed. The result is a change in receptor composition and a dramatic change in function. ARIA promotes the expression of ϵ expression in rat muscle cell lines, and recent data suggest that it suppresses γ expression. This observation provides additional evidence that ARIA does, in fact, operate at the motor endplate. More direct evidence will depend on the development of specific ARIA antagonists. Blocking antibodies and non-toxic effective anti-sense oligonucleotides are needed.

Second, what is the spectrum of ARIA action at the neuromuscular junction? Voltage gated sodium channels are concentrated in the depths of the postsynaptic folds. Significantly, ARIA increases the peak inward Na^+ current and it increases the number of ^3H saxitoxin binding sites in chick muscle.

In addition to their effect on AChR synthesis, motor axons promote the aggregation and immobilization of receptors that are exposed on the muscle surface before nerve contact is established. ARIA does not promote AChR aggregation. Agrin, a protein purified from Torpedo electric organ basement membrane does promote the aggregation of muscle AChRs. Do ARIA and agrin act together to form and stabilize AChRs in the postsynaptic membrane?

Third, what are the effects of ARIA on central and peripheral neurons and glia? Our first experiments are designed to test the effect of ARIA isoforms on neuronal nicotinic AChRs as assayed in the medial habenula, hippocampus and autonomic ganglia. ARIA does promote tyrosine phosphorylation of a 185 kD protein in these cells. Recent experiments indicate that a different form of ARIA can regulate neuronal nicotinic receptors more effectively than the original isoform.

In situ hybridization shows that ARIA is expressed in the subventricular zone of the telencephalon, in the external granule layer of the cerebellum and in the olfactory epithelium. The appearance in such germinal zones raises the possibility that ARIA influences the proliferation of early neuronal precursors or the maturation and survival of postmitotic neurons. Early experiments indicate that ARIA does influence the rate of process outgrowth and the proliferation of PC12 cells - grown in the absence of NGF.

Fourth, what is the mechanism of ARIA action? Intracellular pathways that signal ARIA binding and, ultimately, regulate receptor gene expression remain to be investigated.

THE FUNCTION OF 43K RAPSYN IN SYNAPSE FORMATION AND STABILIZATION

Merlie, J.P., Apel, E.D., Gautam, M., Maimone, M.M., Mudd, J., Nichol, M.C., Noakes*, P.G., Sanes*, J.R.

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In studies aimed at defining the mechanisms that underlie neuromuscular synapse formation, we have focused considerable attention on a 43kDa peripheral membrane protein. Although frequently referred to simply as 43k, we have named this protein rapsyn for receptor associated protein at synapses and the gene *Rapsn* (1) in accordance with the guidelines for gene nomenclature. An abundant component of postsynaptic membranes purified from Torpedo electric organ, 43k rapsyn was recognized early as a potential structural element in the post-synaptic apparatus whose function might be to organize acetylcholine receptors into high density clusters. Several lines of evidence supported this view (see refs. 2 and 3 for review): First, 43k rapsyn was found in stoichiometric amounts with AChR itself. At the ultrastructural level, 43k rapsyn and AChR were found to colocalize precisely suggesting a direct interaction between the two molecules. Indeed, chemical cross-linking revealed the β subunit of the AChR to be within a few angstroms of 43k rapsyn. Finally, extraction of 43k rapsyn and other peripheral membrane components from AChR rich membranes increased the mobility of AChR within the membrane. A molecule with properties similar to 43k rapsyn was identified at murine neuromuscular synapses and at clusters of AChR on the surface of muscle cells in tissue culture. These findings taken together suggested that 43k rapsyn was an important component of the mechanism involved in AChR clustering and organization in the post-synaptic membrane.

Our own work with 43k rapsyn began when peptide sequencing of the Torpedo protein (4) allowed the molecular cloning and complete sequence determination (5). Unique in the sequence data base, the primary structure itself has led to few clues to the mechanisms involved in AChR clustering. However, comparison of the Torpedo, mouse (6) and Xenopus (7) sequences has identified several highly conserved regions that may play particularly important roles in synapse formation. Most importantly, the availability of full length coding sequence for mouse 43k rapsyn has permitted the study of function by expression of recombinant protein in non-muscle cells. Thus, it has been shown that AChR expressed in Xenopus oocytes (8) and transformed quail fibroblasts (9) are uniformly distributed on the cell surface. When coexpressed with 43k rapsyn, AChR are clustered into high density patches on the cell surface; patches on oocytes appear to be much smaller than those in fibroblasts, but both results suggest that 43k rapsyn is directly involved AChR clustering. Double labeling showed that, just as in synaptic membrane and endogenous AChR clusters in cell culture, the 43k rapsyn and AChR were precisely colocalized in the heterologous cell systems. When expressed in the absence of AChR, 43k rapsyn formed identical membrane associated patches suggesting that 43k rapsyn determines the domain structure, and that AChR is organized by these structures. Further experiments showed that AChR diffusely distributed on the membrane surface can be clustered by 43k rapsyn expressed after the synthesis and membrane insertion of the receptors themselves (10). Thus, the organization of AChR clusters can occur in response to the synthesis or regulated clustering of 43k rapsyn.

The recombinant expression system has been used to dissect regions of the 43k rapsyn and AChR molecules involved in the clustering process. These studies have implicated the myristoylated N-terminal in plasma membrane targeting, and a region in the middle of the primary sequence in the interaction with AChR. The C-terminal also seems to play a critical role in AChR clustering (11); point mutations in this region that disrupt a Zn⁺⁺ binding domain

reduce AChR cluster formation (12). Expression of individual AChR subunits led to the surprising observation that all 5 subunits are able to interact with and be clustered by 43k rapsyn (13). Thus, each AChR pentamer has the potential to interact with five 43k rapsyn molecules, a stoichiometry that allows for extensive cross-linking. It is not yet known whether a single 43k rapsyn can interact with more than one AChR subunit, or whether this type of multivalent cross-linking requires additional protein components.

The relative immobility of the AChR-43k rapsyn clusters in muscle cells (10) suggests an interaction with the underlying cytoskeleton. In pursuing this possibility, we have made the observation that large endogenous AChR clusters in C2 myotubes are associated with the dystrophin related protein, DRP/utrophin. This finding is particularly intriguing in light of the recent evidence that dystroglycan serves as a receptor for the nerve-derived AChR cluster-inducing molecule, agrin (see ref. 14 for review). Thus, in addition to providing a link to the cytoskeleton, this association suggests that the induction of AChR-43k rapsyn clustering by agrin may be mediated by DRP/utrophin and the associated glycoprotein complex.

To study the involvement of 43k rapsyn in the formation and maintenance of the synapse, we have used targeted mutagenesis to create mice lacking a functional *Rapsn* gene. The first homozygous mutants of these new lines of mice were born alive, but died within 24 hrs of birth. Analysis of the structure and function of the mutant 43k rapsyn deficient synapses, now in progress, should give us a new perspective on the role of 43k rapsyn in synaptogenesis.

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*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

TUESDAY, NOVEMBER 8, 1994

(AFTERNOON, SECOND SESSION)

SYNAPTOGENESIS AND DEVELOPMENT II

Chair: Dr. A. Karlin; Co-Chair: Dr. D.O. Frost

- | | | |
|-------|---------------|--|
| 15:30 | Poo, M.-m. | Activity-Dependent Modulation of Developing Neuromuscular Synapses |
| 16:00 | Bloch, R.J. | A Spectrin-Based Membrane-Skeleton Involved in AChR Clustering |
| 16:30 | Oppenheim, R. | Activity-Dependent Differentiation and Survival of Developing Signal Motoneurons |
| 17:00 | Barde, Y.-A. | Regulation of BDNF mRNA Levels During the Development of Chick Visual System |

Note: Proceed to the Maryland Science Center, across the Inner Harbor. Refreshments begin at 18:00.

ACTIVITY-DEPENDENT MODULATION OF DEVELOPING NEUROMUSCULAR SYNAPSES

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Early synaptic activity is known to modulate the pattern of nerve connections in the developing nervous system, but the cellular mechanism responsible for the activity-dependent modulation is largely unknown. We have developed a simple culture system of isolated developing spinal neurons and muscle cells to investigate the cellular and molecular processes underlying activity-dependent modulation of synaptic efficacy and connectivity. Embryonic spinal neurons and myotomal muscle cells were obtained by dissociation of neural tube tissue of 1-d old *Xenopus* embryos. The cells were plated on clean glass coverslips and cultured at room temperature for 1-d prior to the experiment.

In the first series of experiments, we examined the activity-dependent modulation of synapses made by two presynaptic spinal neurons co-innervating the same postsynaptic muscle cell. The efficacy of the synaptic inputs made by the two neurons were assayed by measuring the amplitude of evoked postsynaptic currents (EPCs) in the muscle cell when the presynaptic neuron was stimulated to fire action potentials at low-frequency. We found that tetanic stimulation of one of the two presynaptic neurons at 2 Hz for a period of 50 sec resulted in immediate depression of synaptic efficacy of the unstimulated neuron (Lo and Poo, 1991). Such heterosynaptic depression was found to persist for as long as the recording was made (up to 1.5 hr). The depression can be induced when the myocyte was recorded in either current- or voltage-clamp ($V_c = -70$ mV) condition during the tetanus, suggesting that postsynaptic depolarization is not required for the heterosynaptic depression (Lo and Poo, 1994). The predominant factor in the induction of heterosynaptic depression appears to be postsynaptic activation of ACh receptors, rather than presynaptic interaction between the tetanized and un-tetanized nerve terminals, since repetitive postsynaptic activation was induced by iontophoretic application of ACh pulses on the surface of the singly-innervated myocyte would effective in inducing synaptic depression and prevention of postsynaptic rise in Ca^{2+} by postsynaptic loading of Ca^{2+} buffer BAPTA abolished the ACh-induced depression (Dan and Poo, 1992a). Similar blockade of heterosynaptic depression in the doubly-innervated myocyte was also observed after the BAPTA application in the postsynaptic cell (Lo and Poo, 1994), again indicating that Ca^{2+} influx into the postsynaptic myocyte is responsible for the induction of the synaptic depression. The notion that Ca^{2+} influx through the ACh channels provides the inductive signal for synaptic depression is consistent with the observation that synaptic depression can be induced when the myocyte was voltage-clamped at resting potential throughout the experiment, since ACh-induced opening of ACh channels allows substantial amount of Ca^{2+} influx through the channel even under voltage-clamp conditions. However, we noted that when repetitive stimuli were applied in the current-clamp condition, a slightly higher synaptic depression was induced (Lo and Poo, 1994). Depolarization-induced opening of myocyte Ca channels may thus contribute in part to the synaptic depression by increasing the amount of Ca^{2+} influx into the postsynaptic cell. This notion was further supported by the finding that when depolarizing current pulses were injected into the myocyte, in the absence of ACh channel activation, a small but significant depression of synaptic response was indeed observed after 100 pulses of repetitive depolarizations (Lo et al., 1994).

The mean amplitude and the profile of amplitude distribution of spontaneous synaptic currents showed no significant change following heterosynaptic depression or ACh-

induced depression of the evoked synaptic currents, suggesting that postsynaptic ACh receptor density or sensitivity was not affected. Furthermore, analysis of the coefficient of variation in the amplitude of evoked responses also suggested that postsynaptic changes in ACh sensitivity is unlikely to be the sole cause of synaptic depression (Dan and Poo, 1992a; Lo and Poo, 1994). Taken together, these results suggest that both heterosynaptic and ACh-induced synaptic depression were predominantly due to a reduction of presynaptic ACh release. Since Ca^{2+} influx into the postsynaptic myocyte is required for the induction of depression, a retrograde signal must be delivered from post- to presynaptic cell to modulate the process of ACh secretion. A different line of experiments also suggested that postsynaptic processes can directly influence the presynaptic transmitter secretion at developing *Xenopus* neuromuscular junctions. Harish and Poo (1992) showed that intracellular loading of a nonhydrolyzable GTP analogue into innervated muscle cells led to a marked increase in the frequency of spontaneous transmitter secretion and a reduction of evoked synaptic responses, while extracellular application of the drug at the same concentration was without effect. Postsynaptic loading of arachidonic acid (AA) produced a similar effect as the GTP analog, and the potentiation effect of both GTP analogs and AA was reversed by an inhibitor of AA metabolism, AA 861. Further studies indicate that a lipoxygenase metabolite, 5-HPETE, appears to be a likely candidate for the retrograde factor involved in modulating ACh secretion. Whether the retrograde signal responsible for these effects was also involved in the heterosynaptic and ACh-induced depression described above remains to be determined.

In addition to the membrane-permeable factors, recent findings on the ability of myocyte to undergo spontaneous and depolarization-evoked exocytosis (Dan and Poo, 1992b) suggests that membrane-impermeable factors may also serve for retrograde interactions through postsynaptic exocytosis. Since this secretion can be increased by elevation of cytosolic Ca^{2+} levels, the Ca^{2+} influx associated with the synaptic current flow provides a natural link between the synaptic activity and localized retrograde signaling. Of particular interests are neurotrophins, a family of protein factors known for their effects on the differentiation and survival of various neuronal populations. Lohof et al. (1993) have shown a rapid potentiation of spontaneous and evoked ACh secretion from *Xenopus* spinal neurons upon treatments with brain-derived neurotrophic factor and neurotrophin-3, two neurotrophins that are known to affect the survival of these neurons. It remains to be determined whether these neurotrophins are indeed produced and secreted by the embryonic muscle cell during synaptic activity.

Presynaptic secretion of neurotrophic factors, which is also likely to accompany synaptic activity, may exert a modulatory action at the developing synapse. Exogenous application of ATP, which is known to be co-released with ACh at neuromuscular junctions, was shown to result in potentiation of spontaneous ACh secretion and a reduction of evoked ACh release (Fu and Poo, 1991). Calcitonin-gene-related peptide (CGRP), a neuropeptide present at presynaptic motor nerve terminal, was shown to enhance the postsynaptic response at developing neuromuscular junctions by increasing the burst duration of embryonic ACh channels (Lu et al., 1993). The effect of CGRP on these ACh channels is mimicked by dibutyryl-cAMP and by cAMP-dependent protein kinase (PKA) and prevented by a specific peptide inhibitor of PKA. Moreover, postsynaptic inhibition of PKA reduced the amplitude and decay time of spontaneous synaptic currents, suggesting that endogenous CGRP may be present as a potentiating factor during the early phase of synaptogenesis.

Since synaptic activity begins immediately after nerve-muscle contact (Evers, et al., 1989), activity-dependent synaptic modulation could be present soon after the onset of synaptogenesis. It is likely that such feedback modulation plays an important role in the

maturation of synapse. The marked effects of electrical stimulation and chemical treatments on developing neuromuscular synapses in cell cultures shown here have illustrated the functional plasticity of the developing synapses over the duration of minutes to hours. A major challenge in the near future is to link these functional changes to structural alterations that underlie the stabilization or elimination of developing neuromuscular connections and to determine whether these findings in cell cultures reflect faithfully developmental processes *in vivo*.

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A SPECTRIN-BASED MEMBRANE-SKELETON INVOLVED IN ACHR CLUSTERING.

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The aggregation, or clustering, of nicotinic acetylcholine receptors (AChR) is one of the earliest events in the formation of the vertebrate neuromuscular junction (NMJ). We have been studying AChR clusters in order to identify and characterize the molecules and macromolecular complexes that participate in postsynaptic differentiation. Our results suggest that a spectrin-based membrane skeleton like that of the human erythrocyte is involved in AChR clustering.

Our experiments utilize cultured rat myotubes, which form large clusters of AChR that closely resemble postsynaptic AChR aggregates at the embryonic NMJ. As these clusters form in regions of the sarcolemma that are firmly attached to the tissue culture substrate they can be isolated by shearing (1) or by extracting with saponin (2). Isolated AChR clusters can be treated biochemically, immunolabeled, or processed for ultrastructural studies.

A spectrin-based membrane skeleton contains spectrin in a stoichiometric complex with peripheral membrane proteins that themselves are linked to one or more integral membrane proteins. Ultrastructural studies of the human erythrocyte (3) indicate that the network is composed of filaments that measure ~30 nm in length and ~6 nm in diameter, and that come together at intersections of 3-4 filaments. AChR clusters share all these features.

Early experiments from this laboratory demonstrated the presence of an unusual form of β -spectrin associated with clustered AChR (4). Quantitation suggested the presence of 4-7 spectrin molecules for every AChR in the intact cluster (4; Table 1). Studies of the receptor-associated 43K and 58K proteins, suggested that each of these are present in amounts approximately equimolar to AChR (5,6; Table 1). Extraction of isolated clusters under different conditions selectively removes some of these proteins from cluster membrane while leaving others associated with AChR (Table 1). These results suggest AChR and spectrin coexist in a structure that has defined stoichiometry and organization.

Additional evidence for a spectrin-based membrane skeleton was obtained in ultrastructural studies of AChR clusters isolated by shearing and processed by quick-freeze, deep-etch, rotary-replication (1,7). These studies revealed the close association of clustered AChR with a network of filaments that measured ~30 nm in length and ~6 nm in diameter, and that formed intersections of 3-4 filaments, very similar to the membrane skeleton of the human erythrocyte.

There are significant differences, however. Unlike the erythrocyte membrane, AChR clusters contain no α -spectrin but they do contain several distinctive proteins. Dystrophin and DRP/utrophin, for example, associate with clustered AChR, although neither alone appears to be present in amounts stoichiometric with the AChR or the 43K protein (Table 1). Both proteins form part of the spectrin-based membrane skeleton, as seen in quick-freeze, deep-etch replicas (8) and in preparation) but only DRP/utrophin codistributes precisely with AChR. Furthermore, extracellular structures are anchored at AChR clusters, probably through a transmembrane attachment to the membrane skeleton. The cytoplasmic portion of dystroglycan, a component of the glycoprotein complex to which dystrophin and DRP/utrophin bind (9-11), is present at AChR clusters and may help to anchor the extracellular matrix (12,13).

The biochemical interactions responsible for anchoring the AChR are still unclear, however. To identify the AChR-associated proteins that are most important for clustering, we microinjected rat myotubes with specific monoclonal antibodies (mAbs), followed by goat anti-

Table 1: Stoichiometry and Susceptibility to Extraction of AChR-Associated Proteins of AChR Clusters

Protein	PBS	Buffer A	CT	pH 11	LIS or urea
	(relative amounts of epitope available to mAb)				
AChR	1	1	1	2	2
43K	1	1	1	0	0
58K	1	1	0	0.3	0
β -spectrin	4-7	2	0	0	0
dystrophin	0.2	0.2	0	0.2	0.2
DRP/utrophin	0.2-0.7	0.2-0.7	0	0.2-0.7	0.2-0.7

Clusters were isolated, treated under the indicated conditions for 5-10 min at RT, fixed, and labeled with monoclonal antibodies specific for each AChR-associated protein, followed by fluorescent anti-antibodies. Relative amounts were estimated by quantitative fluorescence measurements. For details, see refs. (4-6,8).

mouse IgG. MAb 1579, directed against the C-terminal region of the receptor-associated 43K protein, disrupts AChR clusters, whereas mAbs against a more N-terminal epitope of 43K, or against cytoplasmic epitopes of the AChR itself, have no effect. This suggests that the 43K protein -- and specifically its C-terminal region -- is necessary for AChR clustering.

We are currently pursuing these experiments further to evaluate the role in clustering of the other proteins listed in Table 1. We are also characterizing the receptor-associated β -spectrin at the molecular level and studying the role of the spectrin-based membrane skeleton in the formation of central synapses.

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ACTIVITY-DEPENDENT DIFFERENTIATION AND SURVIVAL OF DEVELOPING SPINAL MOTONEURONS

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Neuromuscular activity in the developing embryo begins almost as soon as motoneurons (MNs) make their first provisional synaptic contacts with myotubes. In the chick embryo this begins on embryonic day 4 (E4) when the first neurally mediated movements of the embryo can be observed in the egg. In rodent and human embryos this milestone is reached on about E14 and E60, respectively. In all vertebrates, the onset of this neurobehavioral activity occurs long before the central and peripheral nervous systems are fully differentiated. Proliferation, migration, pathway formation, cell death and synaptogenesis continue in the spinal cord during the early stages of functional activity. Accordingly, it is reasonable to ask whether the embryonic activity contributes in any way to the differentiation and organization of the emerging nervous system. We have been particularly interested in the role of neuromuscular activity in the regulation of MN differentiation and survival in the developing avian embryo in ovo.

The first indication that activity was important in this system came from studies in which neuromuscular transmission was blocked chronically with agents such as curare and α BTX beginning on E4-5 and continuing for several (5-10) days. Whereas, normally about 50% of spinal MNs undergo programmed cell death (PCD) during this period, following activity blockade cell death failed to occur such that these embryos had thousands of excess MNs. Further examination revealed that despite a retardation of muscle differentiation in this situation, the MNs had differentiated normally based on morphological, biochemical and functional criteria. All of the excess MNs projected axons to and formed synapses on skeletal myotubes and myofibers, resulting in hyperinnervation. When the activity blockade was removed, MN numbers and innervation gradually returned to control values. In another, experiment, it was shown that electrical stimulation of limb muscles in ovo resulted in fewer surviving MNs in both control and activity blocked embryos. Taken together, these initial studies strongly indicated that neuromuscular activity in the developing embryo acts to regulate MN survival and differentiation. Further support for this conclusion came from the examination of avian and mouse muscular dysgenic mutants in which neuromuscular activity in the embryo is absent owing to defects in excitation-contraction coupling. These embryos exhibited all of the same perturbations in motoneuron development as seen in activity blocked avian embryos, including increased MN survival and hyperinnervation of muscle. Because the only common feature in all of these situations is the absence of muscle contraction, we conclude that it is muscle activity and not spinal cord activity or neuromuscular transmission that is the critical event responsible for the observed effects on MN development and survival.

Because target derived neurotrophic agents such as NGF are known to regulate the survival and differentiation of many populations of neurons, we next tested the notion that activity may act to regulate the expression, production or secretion of a muscle-derived MN trophic agent. According to this hypothesis, decreased muscle activity (neuromuscular blockade) would increase

trophic factor, whereas increased activity (electrical stimulation) would decrease the amount of trophic factor. Although we cannot entirely exclude this possibility, the evidence is not consistent with this explanation. Extracts of muscle derived from either normally active or activity blocked embryos are equally effective in promoting MN survival *in vitro* and *in vivo*. In contrast, the evidence is more consistent with the notion that activity acts to regulate the interaction of MNs with target muscles by modulating axonal branching and synaptogenesis. For example, activity blockade results in a 2-3 fold increase in intermuscular axon branching/sprouting and synapse formation which may provide MNs with increased access to a muscle-derived trophic agent. If correct, then this suggests that a primary event regulated by muscle activity is the availability of a muscle-associated axon sprouting agent. Preliminary evidence indicates that a strong candidate for this agent is insulin-like growth factor (IGF). IGF is expressed in developing muscle and is upregulated following activity blockade. Inhibition of IGF activity by IGF binding proteins prevents the increased MN survival and axon branching induced by activity blockade and treatment of embryos with exogenous IGF rescues MNs from cell death *in vivo* and promotes MN survival *in vitro*. Although considerably more work is needed to confirm that IGF plays an important biological role in the activity-dependent regulation of MN development and survival, the available evidence is generally consistent with this notion.

In summary, we conclude that the differentiation and survival of developing MNs is regulated by muscle activity. Muscle activity appears to regulate the availability of a sprouting agent that acts to control branching and synapse formation by MN axons. Because target-derived survival promoting trophic agents are thought to interact with innervating neurons via synaptic terminals, the net effect of muscle activity is to regulate access of MNs to a target-derived trophic agent that is necessary for their maintenance, survival and further differentiation. Whether IGF acts as both a sprouting and survival promoting agent in this situation, or whether the two functions are mediated by separate molecules is presently unknown.

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REGULATION OF BDNF mRNA LEVELS DURING THE DEVELOPMENT OF THE CHICK VISUAL SYSTEM

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During development, neurons require specific signals if they are to survive past the time of naturally occurring cell death. Soon after the last mitosis, when axon terminals first contact their target cells, neurons seem to be exquisitely sensitive to the presence of target-derived signals that are able to stop the death program operating in most embryonic neurons. In the peripheral nervous system, nerve growth factor (NGF) has been demonstrated to be expressed in the target cells of the neurons that need this protein for survival. In addition, both at the mRNA and protein levels, the quantities of NGF in the target field correlate with the density of innervation, suggesting that the limited quantities of protein secreted by the target cells play an important role in determining the degree of innervation. If, for example, the NGF gene is placed under the control of a keratin promoter, the density of sensory innervation of the skin, as well as the number of neurons in the corresponding ganglia, is significantly increased, indicating that the potential normally present in developing peripheral ganglia is not fully exploited¹. In the peripheral nervous system, it seems that the transcription of the NGF gene is not regulated by ingrowing axons, as complete denervation (by ablation of the neural crest giving rise to the peripheral sensory ganglia) does not change the onset or the extent of the transcription of the NGF gene².

It is now apparent that NGF was the first member of a gene family to have been discovered, and the other members of the NGF, or neurotrophin, family are also all able to prevent the death of a variety of neurons in culture. For some of them, it appears very likely that they serve a similar function also *in vivo*, as gene deletion and antibody deprivation experiments lead to severe neuronal losses in the peripheral nervous system. Conversely, at least one non-NGF neurotrophin, brain-derived neurotrophic factor (BDNF), is able, like NGF, to prevent normally occurring neuronal death in peripheral sensory ganglia, as well as of some motoneurons in the spinal cord and of the isthmo-optic nucleus. But at this stage, still remarkably little is known about the role of neurotrophins during normal development in the central nervous system, and the data available indicate that it is unlikely that the neurotrophins (including NGF) play a simple survival role, similar to that established in the peripheral nervous system. For example, in transgenic mice whose functional NGF or BDNF gene has been deleted by homologous recombination, no massive cell death (beyond that normally seen during development) could be observed in a series of neurotrophin-responsive structures, such as the basal forebrain cholinergic neurons, the spinal or facial motoneurons, the dopaminergic neurons of the substantia nigra, or the retinal ganglion cells^{3,4,5}. This is intriguing, as all these groups of CNS neurons show a response to neurotrophins either *in vitro* or after section (or lesion) of their axons *in vivo*.

We are interested to learn about the role of BDNF during normal development of CNS neurons. We used for our studies the visual system of the chick to examine the regulation of the expression of the BDNF gene with regard to events related to target innervation. The avian retina and the optic tectum are particularly convenient structures to study, as the optic tectum represents the only major target of the retinal ganglion cells. In addition, numerous previous studies have established with precision the timing of the arrival of the first retinal ganglion cell axons, as well as the timing and degree of elimination of retinal ganglion cells, both during normal development and after ablation of the tectum. Also, we exploited the possibility offered by bird embryos to be manipulated during development. Finally, a previous study had established that *in vitro*, chick retinal ganglion cells respond to BDNF: when dissociated from E11 retinae, few will survive in the absence of BDNF.

To study the expression of the BDNF gene, we set up a simple and quantitative PCR-based quantification method⁶. This assay makes use of a mutated RNA standard that differs from the endogenous mRNA by a single base, thus creating a restriction site not present in the wild-type message. This allows the standard (added at the time of tissue homogenisation) to be distinguished from the endogenous mRNA at the end of the amplification procedure. This method, which involves quantification using a single reaction tube and low cycle numbers, was validated by comparing the copy numbers determined with those obtained with quantitative Northern blot analysis (performed with tissues containing relatively high copy numbers). Curiously, the BDNF mRNA levels were found to be high in the tectum at E4, which is 2 days before the first retinal ganglion cell axons arrive at the tectum. This might be related to the development of the tecto-bulbar tract, which express high levels of trkB (a BDNF receptor) at this stage. At E6, the lowest BDNF mRNA levels were determined. Only 300,000 copies per mg tissue were found, and assuming that the tectum has a cellular density similar to that found in the retina, for which cell numbers are available, this would correspond to about one copy of BDNF mRNA for every 5 cells. A strong increase (of more than 10-fold) follows until E11, and thereafter the levels decrease by about 30% until E17. We were interested to see if the massive increase in copy numbers seen in the tectum after E6 can be correlated with the arrival of the retinal ganglion cell axons. In order to explore this possibility, the right optic stalk was severed at E4 to prevent tectal innervation. At E7, the left, non-innervated tectal was compared with the innervated side. A reduction by 43% in the operated side was observed when compared with the contralateral, non-operated side. In order to test for a possible role of electrical activity, tetrodotoxin was injected into one eye at E6. Comparison of BDNF mRNA levels in both tecta at E7 showed a reduction in the tectum contralateral to the injected side by 32%. This suggests that a significant portion of the effects resulting from the absence of tectal innervation can be accounted for by action potential-mediated events taking place in the retinal ganglion cells. These results indicate that the first projecting ganglion cells are electrically active, and that this activity has measurable consequences on the steady-state levels of BDNF mRNA in CNS target cells.

BDNF mRNA was detected not only in the tectum, but also in the retina at E6, the first time point investigated. The levels were found to increase until E11, and were maintained until E17, with a reduction to about half until adulthood. While we could not localise BDNF mRNA anywhere in the chick visual system by *in situ* hybridisation, immunostaining with an antiserum

raised against a BDNF peptide, as well as against BDNF, revealed BDNF-immunoreactivity in the retinal ganglion cells themselves. This immunoreactive material does not seem to result from the retrograde accumulation of BDNF derived from the tectum, since optic stalk transection did not affect the immunostaining observed at E8 and E11 (i.e. before the time when retinal ganglion cell disappear as a result of the optic stalk transection). It thus appears that somewhat surprisingly, a probable site of synthesis of BDNF in the retina might be in the retinal ganglion cell themselves. While the functional consequences of these findings are unclear at present, it is interesting to note that at least in some neurons of the peripheral sensory ganglia, BDNF mRNA and/or immunoreactivity has been noted.

In summary, our results indicate that the BDNF gene is expressed during the development of the chick visual system both in the retina and the tectum. During the initial phase of tectal innervation, the gene is expressed at extremely low levels, and the steady state levels of BDNF mRNA in tectal cells are under the influence of the retinal ganglion cell axons. It appears likely that the neurotransmitter(s) released by the electrically active ganglion cells play a role in this regulation. In view of recent results obtained with BDNF $-/-$ mice by others^{4,5}, it appears unlikely that a major function of BDNF would be to regulate the survival of retinal ganglion cells in the retina during normal development (unlike in the peripheral nervous system). Amongst other possibilities for the function of BDNF might be the regulation of branching of the axon terminals, of the motility of the growth on the tectum, of the release of neurotransmitters or of the expression of various peptides by the ganglion cells. There are examples for some of these possibilities with BDNF in *in vitro* systems.

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*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
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NOTES

TUESDAY, NOVEMBER 8, 1994

18:45-20:15

THE JOHN C. KRANTZ, JR. LECTURE

PROF. B. SAKMANN

*Molecular Basis and Functional Consequences of the γ/ϵ -AChR Subunit Switch
at the Rat Neuromuscular Junction*

MOLECULAR BASIS AND FUNCTIONAL CONSEQUENCES OF THE γ/ϵ -AChR SUBUNIT SWITCH AT THE RAT NEUROMUSCULAR JUCTION

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Most receptor channels are heterooligomers composed of several different subunits. Receptor channels operated by transmitters released at fast-transmitting synapses such as acetylcholine (ACh), γ -aminobutyric acid (GABA), glycine (Gly), or glutamate (Glu) are thought to form a channel "superfamily" because they share structural and functional properties. The expression of channel subtypes in muscle fibers or neurons often changes during development. This may suggest that the multitude of channel subtypes is essential for cell function at a particular developmental stage or for a tissue-specific function.

Nicotinic AChR channels of skeletal muscle (endplate channels) represent a relatively simple case because only two functionally different channel subtypes are observed, a *fetal* AChR channel and an *adult* AChR channels consisting of α -, β -, γ - and δ -subunits and α -, β -, δ - and ϵ -subunits, respectively. The expression of these two AChR subtypes is under neural control because the density of the two subtypes in the postsynaptic membrane as well as their cellular location and their functional properties are regulated by the motor nerve during normal synapse formation in developing muscle and during ectopic synapse formation in adult muscle.

Functional differences between *fetal* and *adult* AChR channels are observed in the channels' conductance for monovalent alkali cations, divalent cation permeability and fractional Ca^{2+} conductance and in the gating behaviour. The structural basis of the difference in functional properties is an exchange of γ -subunit present in the fetal type by the ϵ -subunit in the adult AChR. The molecular determinants that specify the different functional properties of the two AChRs are located in the putative transmembrane segments M1 to M4 of the γ - and ϵ -subunit. These segments are encoded by separate exons of the respective genes.

The developmental changes in abundance and cellular localisation of γ - and ϵ -subunit specific mRNAs, measured during synapse formation of normal and ectopic synapses, suggest that differential expression of γ - and ϵ -subunit genes in muscle is under the control of neural factors through a mechanism by which the nerve terminal controls the molecular properties of AChR channels during synapse formation. The mechanisms underlying the γ - and ϵ -subunit switch involve a perinatal local "imprinting" by the nerve, which appears to be mediated by two trophic factors: one that causes permanent expression of ϵ -subunit mRNA in synaptic nuclei, and a second that causes inactivation of γ -subunit gene expression in synaptic and perisynaptic nuclei. Inactivation of the γ -subunit gene in subsynaptic nuclei requires vesicular transmitter release from the motor nerve terminal.

In summary, the localized activation of the ϵ -subunit gene and localized inactivation of the γ -subunit gene in sub- and perisynaptic nuclei by trophic signals from the nerve ending mediate a switch in molecular composition of postsynaptic AChR in developing an adult muscle. One functional consequence of this switch is a larger localized postsynaptic Ca^{2+} inflow in the adult end-plate.

The mechanisms underlying developmental regulation of endplate AChR subtypes could be prototypical for the regulation of functional properties of other heteromeric receptor channels. For example, AMPA- and NMDA-type glutamate receptors in the central nervous system are heteromultimeric channels. Different combinations of subunits are assembled to channels which mediate fractional Ca^{2+} currents varying over a more than fifty fold range. In the central nervous system developmental switches in glutamate receptor subunit expression are observed which could mediate changes in Ca^{2+} inflow through the postsynaptic membrane and which could control changes in synaptic efficacy.

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WEDNESDAY, NOVEMBER 9, 1994

(MORNING, FIRST SESSION)

CNS RECEPTOR FUNCTION AND STRUCTURE I

Chair: Dr. S. Fuchs; Co-Chair: Dr. B. Alger

8:00	Patrick, J.	Cyclophilin-Dependent Expression of $\alpha 7$ Neuronal Nicotinic Acetylcholine Receptor
8:30	Lindstrom, J.	Neuronal Nicotinic $\alpha 3$, $\alpha 4$, $\alpha 7$, and $\alpha 8$ AChRs
9:00	Albuquerque, E.X.	Functional and Structural Aspects of Neuronal Nicotinic Receptors
9:30	Maelicke, A.	Noncompetitive Agonism at Nicotinic Acetylcholine Receptors
10:00	Colquhoun, D.	Comparison of Native Recombinant Receptor Channels: Glutamate and Acetylcholine Receptors
10:30	--	Coffee Break -- Poster Session

Cyclophilin-dependent Expression of $\alpha 7$ Neuronal Nicotinic Acetylcholine Receptor

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Danong Chen, Finn Goldner and Jim Patrick

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Neuronal nicotinic acetylcholine receptors (nAChRs) are expressed in throughout the brain as evidenced by both ligand binding and in situ hybridization studies (1, 2). α -bungarotoxin (α -BTX) binding sites are found on one class of nAChRs that are also known to be widely-distributed in the brain (3). It is now clear that the nAChR subunit $\alpha 7$ constitutes an important component of the receptor that binds α -BTX (4, 5, 6). This subunit forms a homo-oligomeric ligand-gated ion channel when expressed in *Xenopus* oocytes (4, 5). This ligand-gated channel is activated by nicotinic agonists, and shows a pharmacological profile that is characteristic of a nAChR (4, 5, 7). The agonist-induced current through this channel desensitizes rapidly, is inwardly rectifying at positive membrane potentials, and is mainly carried by monovalent cations. There is, however, a very significant divalent cation permeability that suggests a role for this channel in neural plasticity (5, 8). Although the precise subcellular locale of this receptor is unclear it has been speculated that it might be localized to the synaptic terminal and there is evidence that nicotine modifies synaptic transmission between pre and post-synaptic sympathetic neurons in an α -BTX sensitive fashion (9). We have raised polyclonal antibodies against this subunit, and are currently determining its cellular and subcellular distribution in the brain using immunohistochemistry and immuno-electron microscopy.

The fact that the nAChR comprised of $\alpha 7$ subunits is a homo-oligomer makes it particularly well suited for expression studies and we have studied the expression of this receptor in the *Xenopus* oocyte. An initial result of these studies was the observation that expression of the $\alpha 7$ homo-oligomer was sensitive to CyclosporinA suggesting that the peptidyl-prolyl isomerase cyclophilin was required for expression of functional receptors on the surface of the oocyte (10). Cyclophilin is required for expression of functional receptors, α -BTX binding sites, and $\alpha 7$ antigenic determinants. Cyclophilin is not required for expression of the hetero-oligomeric muscle nicotinic receptor. Co-expression of the $\alpha 7$ subunit with the muscle nicotinic receptor beta, gamma, and delta subunits removes the requirement for cyclophilin (11). The delta subunit alone can supply this function. The requirement for cyclophilin is not unique to the $\alpha 7$ homo-oligomeric receptor; expression of the 5HT3 receptor (12) is also sensitive to CyclosporinA.

The mechanism by which cyclophilin participates in expression of a homo-oligomeric receptor is not known. In principal it could contribute either its chaperone activity or its peptidyl-prolyl isomerase activity. In one case it might stabilize subunits during the formation of multimers and in the other case it might be required for the generation of the proper isomer of a critical proline residue in $\alpha 7$ subunit. We are testing these alternatives by co-expression of the $\alpha 7$ with cyclophilins that have lost certain functions by mutagenesis.

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NEURONAL NICOTINIC $\alpha 3$, $\alpha 4$, $\alpha 7$, and $\alpha 8$ AChRs

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There are three branches of the nicotinic acetylcholine receptor (AChR) gene family: 1) muscle type AChRs, 2) neuronal AChRs which do not bind α bungarotoxin (α Bgt), and 3) neuronal AChRs which do bind α Bgt. The muscle-type AChRs of *Torpedo* electric organ have the subunit composition $(\alpha 1)_2\beta 1\gamma\delta$ and are the archetype for structural studies of this gene family. Neuronal AChRs which do not bind α Bgt are thought to be composed of combinations of $\alpha 2$ – $\alpha 6$ subunits with $\beta 2$ – $\beta 4$ subunits. In mammalian brains AChRs with the subunit composition $(\alpha 4)_2(\beta 2)_3$ account for >90% of the high affinity binding sites for ACh and nicotine, whereas AChRs composed of $\alpha 3$, $\alpha 5$, and $\beta 4$ or $\beta 2$ subunits appear to predominate in retina and ganglia and exhibit somewhat lower affinity for ACh and nicotine. Neuronal AChRs which bind α Bgt contain $\alpha 7$ or $\alpha 8$ subunits, but may also contain other structural subunits which have not yet been identified. We will describe our recent results with native and expressed neuronal AChR subtypes which contain $\alpha 3$, $\alpha 4$, $\alpha 7$, and $\alpha 8$ subunits.

$\alpha 4\beta 2$ AChRs

This subtype is the best characterized neuronal AChR from a structural point of view. It is suspected that sequence homologies between subunits in the gene family reflect homologous shapes that cause all neuronal AChRs to have a pentameric arrangement of subunits similar to that known for muscle AChRs. However, it is only in the case of $\alpha 4\beta 2$ AChRs where N-terminal amino acid sequencing of subunits from purified AChRs has been used to identify subunit composition with certainty, and only in this case that studies of expressed subunit cDNAs have defined the stoichiometry as $(\alpha 4)_2(\beta 2)_3$.

The physiological roles of $\alpha 4\beta 2$ AChRs are not well characterized. Evidence from several laboratories suggests that many $\alpha 4\beta 2$ AChRs are located presynaptically where their physiological role may be to modulate the release of transmitters such as dopamine.

Chronic exposure of animals or tobacco smokers to nicotine causes an increase in brain $\alpha 4\beta 2$ AChRs which is associated with development of tolerance and addiction. We have studied nicotine-induced upregulation of $\alpha 4\beta 2$ AChRs expressed in *Xenopus* oocytes and permanently transfected fibroblasts. In these systems nicotine concentrations similar to those found in smokers cause an increase in the amount of AChR to an extent and with a time course similar to that observed in brain. Nicotine and other agonists appear to cause upregulation without requiring ion flow through the AChR channel because: 1) chronic exposure to nicotine appears to result in a permanently desensitized conformation of the AChR, and 2) because the noncompetitive antagonist mecamylamine also causes upregulation and is partially additive with nicotine in causing upregulation. Mecamylamine is an open channel blocker which is a more effective blocker in the presence of nicotine. Nicotine causes a decrease in the rate of internalization and destruction of $\alpha 4\beta 2$ AChRs which is sufficient to account for the extent of upregulation which is observed. Thus, agonists and some noncompetitive antagonists appear to induce a conformation of $\alpha 4\beta 2$ AChRs which is turned over more slowly, causing a net increase in AChRs which retain the ability to bind ligands but are functionally inactive. This accumulation of functionally inactive AChRs can account for tolerance as a result of chronic exposure to nicotine.

$\alpha 3$ AChRs

$\alpha 3$ AChRs have been best characterized by Berg and co-workers in chick ciliary ganglia. $\alpha 3$ AChRs in these cells have a classic postsynaptic role. These AChRs appear to include $\alpha 5$ and $\beta 4$ subunits, but the cells also make $\beta 2$ and $\alpha 7$ subunits.

We have been studying human $\alpha 3$ AChRs in the cell line SH-SY5Y and expressed from cRNAs in *Xenopus* oocytes using combinations of $\alpha 3$, $\beta 2$, $\beta 4$ and $\alpha 5$ subunits. mAbs to the main

immunogenic region on the extracellular surface of $\alpha 1$ subunits crossreact with similar sequences on $\alpha 3$ and $\alpha 5$ subunits. This may be relevant to the autoimmune response to muscle AChRs in myasthenia gravis, since the thymus (which may be involved in the initiation of the autoimmune response) contains both $\alpha 1$ and $\alpha 3$ AChRs. Although evidence has been presented by Berg and co-workers that $\alpha 5$ is a component of ganglionic AChRs in combination with $\alpha 3$ and $\beta 4$ subunits, when co-expressed in oocytes $\alpha 5$ decreases the surface expression of functional $\alpha 3\beta 4$ or $\alpha 3\beta 2$ AChRs, but some $\alpha 5$ does make it to the surface in combination with the other subunits.

$\alpha 7$ and $\alpha 8$ AChRs

$\alpha 7$ and $\alpha 8$ cDNAs were first cloned from chickens, but now $\alpha 7$ has also been cloned from rats and humans. We found that there are substantial pharmacological differences between native chick $\alpha 7$ and $\alpha 8$ AChRs and between $\alpha 7$ and $\alpha 8$ homomers expressed in *Xenopus* oocytes, but that the channel properties of the homomers are identical. Both $\alpha 7$ and $\alpha 8$ homomers form rapidly desensitizing, Ca^{++} -selective channels. $\alpha 7$ homomers are expressed on the surface of oocytes as efficiently as are $\alpha 1\beta\gamma\delta$ or $\alpha 4\beta 2$ AChRs. $\alpha 8$ homomers are expressed on the oocyte surface much less efficiently, presumably reflecting their greater dependence upon co-assembly in vivo with structural subunits (which are as yet unknown). $\alpha 7$ AChRs and $\alpha 7$ homomers have higher affinity for α Bgt than do $\alpha 8$ AChRs or $\alpha 8$ homomers. Conversely, $\alpha 8$ AChRs have higher affinity for small cholinergic ligands. Expression of mosaics between $\alpha 7$ and $\alpha 8$ reveal that some of the 5 amino acids which differ between $\alpha 7$ and $\alpha 8$ in the region 179-208 account for these pharmacological differences. Human $\alpha 7$ cloned from SH-SY5Y cells expressed as homomers have pharmacological properties similar, but not identical to native human $\alpha 7$ AChRs, perhaps reflecting the presence of an unknown structural subunit in the native $\alpha 7$ AChR. The most striking pharmacological difference between human and chick $\alpha 7$ homomers is that DMPP is virtually an antagonist (<3% partial agonist) on chick $\alpha 7$, but the most potent agonist on human $\alpha 7$.

In order to gain insight into the functional roles of AChR subtypes, they have been localized histologically in brain and retina. mAbs to AChR subunits have been used to immunohistologically localize AChR subunits in collaboration with Luiz Britto, Kent Keyser, Harvey Karten, Manuel Criado, Jose Juiz, and Larry Swanson.

An example of an unusual synaptic mechanism involving AChRs is provided by hair cells. Paul Fuchs and co-workers found that chick cochlear hair cells have an AChR which is blockable by α Bgt and which permits entry of Ca^{++} that acts as a second messenger to trigger a Ca^{++} -sensitive K^+ channel resulting in a net prolonged inhibitory response. Because many of the basic properties of this AChR resemble $\alpha 7$ AChRs, we looked for $\alpha 7$ AChRs in cochlea. Cochlea contain $\alpha 7$ mRNA. mAbs to $\alpha 7$, but not $\alpha 8$, $\alpha 3$, $\alpha 4$ or $\beta 2$ could immunoprecipitate $^{125}I\alpha$ Bgt-labeled AChRs from cochlea. These have the pharmacological properties of brain and retina $\alpha 7$ AChRs. Individually dissected hair cells are labeled by mAbs to $\alpha 7$. These hair cells may also contain some other AChR subunits. Nicotine is an agonist on $\alpha 7$ homomers but is reported to be an antagonist on hair cells. Thus the AChR studied by Fuchs and co-workers may include $\alpha 7$ subunits in combination with structural subunits that affect their pharmacological properties, or in addition to $\alpha 7$ AChRs an additional AChR maybe present to account for all of the pharmacological properties observed by Fuchs and co-workers. In any case, the unusual sort of synaptic mechanism observed in hair cells, where Ca^{++} acts as a second messenger, could provide an example of the potentially novel and as yet unknown sorts of mechanisms in which $\alpha 7$ AChRs may participate.

Central nervous system processes associated with nicotinic AChRs that may be important as pharmacological targets are only beginning to be characterized. A potential role in attention, learning and memory is suggested by the loss of AChRs observed in Alzheimer's and Parkinson's diseases and the beneficial effects of nicotine on some cognitive tasks. Nicotinic AChRs may also be involved in perception of pain. Epibatidine is a nicotinic AChR ligand which is much more potent than morphine as an analgesic. We found that epibatidine is a very potent agonist at several types of neuronal AChRs, but that it has negligible affinity for muscle AChRs. It is by far the

highest affinity ligand which we have observed for binding to human $\alpha 3$ AChRs ($IC_{50} = 1pM$) and has lower affinity for $\alpha 7$ AChRs ($IC_{50} = 20nM$). As an agonist epibatidine is 100-1000 fold more potent than ACh or nicotine for $\alpha 3$, $\alpha 4$, $\alpha 7$, and $\alpha 8$ AChRs. Epibatidine is likely to prove to be a very useful ligand for structural and functional studies, may provide a lead compound for new types of analgesics, and may help to reveal new physiological roles for nicotinic AChRs.

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FUNCTIONAL AND STRUCTURAL ASPECTS OF NEURONAL NICOTINIC RECEPTORS.

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The diversity of neuronal nAChRs, in addition to their possible involvement in such pathological conditions as Alzheimer's disease, has directed our research towards the characterization of these receptors in various mammalian brain regions. Our studies have relied on electrophysiological, biochemical, and immunofluorescence techniques applied to cultured and acutely dissociated hippocampal neurons. In hippocampal neurons, a nicotinic agonist can evoke three types of whole-cell currents, which have been classified as types IA, II, or III according to their kinetic properties and their sensitivities to pharmacological agents. Type IA currents are sensitive to blockade by α -bungarotoxin (α -BGT) or methyllycaconitine, inactivate rapidly, and have low sensitivity to ACh ($EC_{50} = 130 \mu M$). Type II currents are sensitive to blockade by dihydro- β -erythroidine, inactivate slowly, and have high sensitivity to ACh ($EC_{50} = 2 \mu M$). Type III currents are sensitive to blockade by mecamylamine and inactivate very slowly. Composite currents, referred to as IB and made up of a combination of type IA and II currents, can be found in a few hippocampal neurons, indicating that more than one nAChR subtype can be expressed in a single neuron (Fig. 1A)¹⁻⁴. The peak amplitude of IA currents tends to rundown when the internal solution is devoid of ATP-regenerating compounds, and phosphocreatine in particular⁵. In addition, rectification of IA currents is observed only when Mg^{2+} is added to F^- -free internal solution⁵. The characteristics of nicotinic currents activated in hippocampal neurons indicated that these neurons express (i) an $\alpha 7$ -subunit-bearing nAChR, which subserves type IA currents, (ii) an $\alpha 4\beta 2$ nAChR, which subserves type II currents, and (iii) an $\alpha 3\beta 4$ nAChR, which subserves type III currents. By means of *in situ* hybridization, mRNAs coding for $\alpha 7$, $\alpha 4$, and $\beta 2$ nAChR subunits have been found in hippocampal neurons⁵. The single channels that account for type IA currents inactivate fast, have a brief lifetime ($\sim 110 \mu s$ at $-60 mV$) and a high conductance (73 pS), whereas channels that account for type II currents have a long lifetime and a low conductance (Fig. 1B)⁶. The ion selectivity of α -BGT-sensitive hippocampal nAChRs was investigated by measuring reversal potentials (V_R) of ACh-induced IA currents in physiological solutions of various ionic compositions. Using a Goldman-Hodgkin-Katz equation for V_R shifts in the presence of Ca^{2+} , P_{Ca}/P_{Cs} 's of α -BGT-sensitive hippocampal nAChR and of NMDA-gated ion channels were found to be about 6 (Fig. 1C) and 10, respectively⁷. Thus, the native α -BGT-sensitive hippocampal nAChR channel is considerably permeable to Ca^{2+} although not as much as the NMDA channel. Since under physiological conditions IA currents show inward rectification, $\alpha 7$ -bearing nAChRs may mediate a sharp rise in intracellular Ca^{2+} levels that is independent of membrane depolarization and maximal at near-resting membrane potentials. In cultured olfactory bulb neurons, nicotinic agonists evoke a single type of whole-cell current, *i.e.* type IA⁸. Our most recent studies have shown that there is an ACh-insensitive nAChR site through which the nAChR channel can be activated. Initially, it was demonstrated that physostigmine (PHY) could directly interact with the muscle nAChR as an agonist and an open-channel blocker^{9,10}. Subsequently, the agonist effect of PHY was observed in several other preparations, each of which expressed different types of nAChRs. PHY, as well as the muscle relaxant benzoquinonium (BZQ) and the anticholinesterase galanthamine (GAL) can activate the nAChRs expressed on frog muscle fibers, *Torpedo* electroplax, hippocampal neurons, M10 fibroblasts, and clonal pheochromocytoma (PC12) cells. The most important findings, however, were that (i) the nAChR-specific monoclonal antibody FK1 could block the agonist actions of these three compounds, without affecting those of ACh, and (ii) competitive nicotinic antagonists had no effect on the agonist actions of PHY, GAL, and BZQ, but could block those of ACh, thus indicating that BZQ, GAL, and PHY could activate the nAChR channel by binding to a site distinct from that for ACh¹¹⁻¹⁶. By means of photoaffinity labeling, the region including and surrounding the residue Lys-125 on the *Torpedo* nAChR α subunit was found to bear the PHY-binding site¹⁷. This residue is not situated

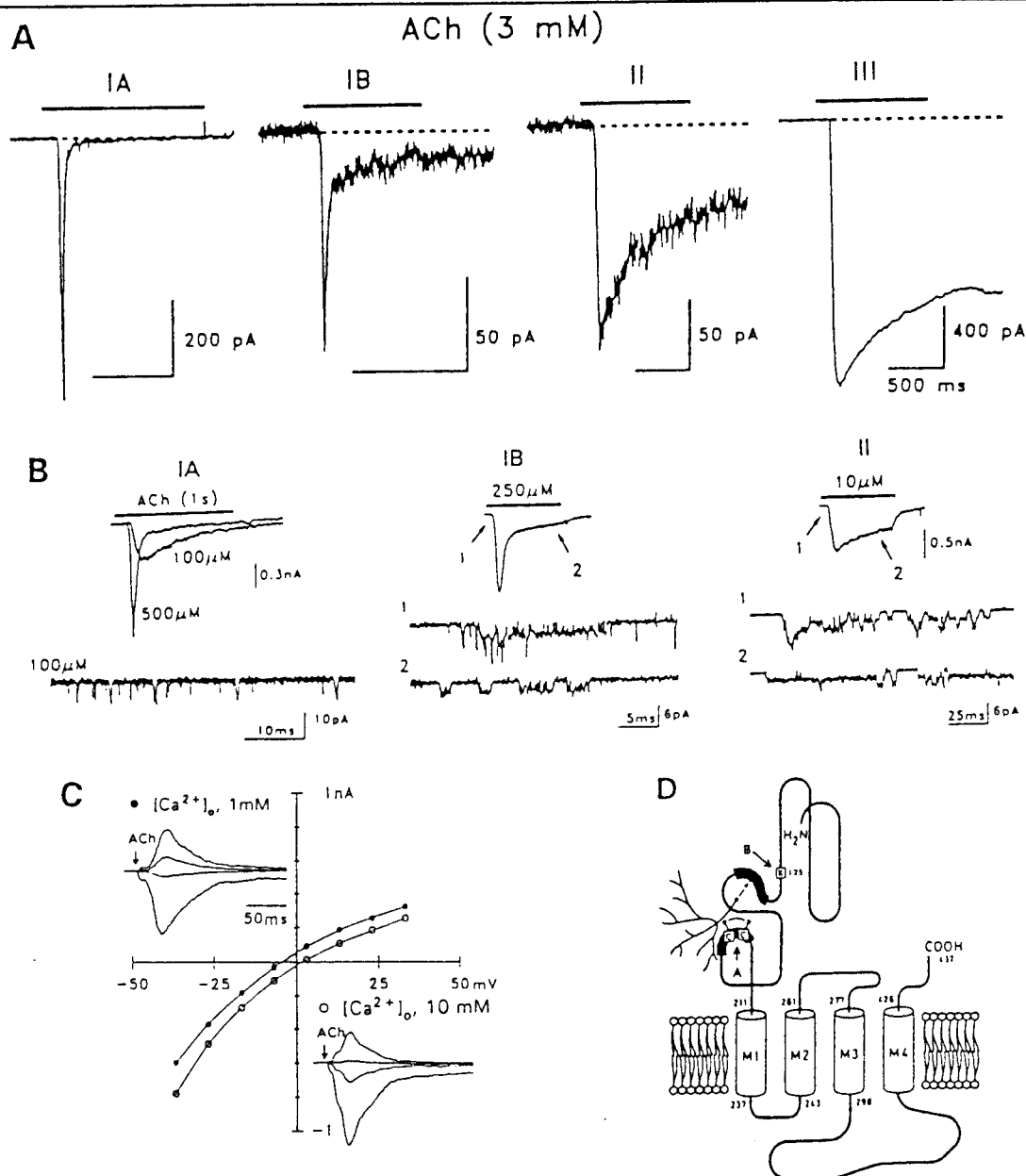


Fig. 1. **A.** Sample recordings of ACh (3 mM)-evoked currents in a 20-day-old cultured hippocampal neuron held at -56 mV. **B. Top traces:** Sample recordings obtained from three hippocampal neurons held at -60 mV showing type IA, IB, or II current activated by ACh. **Bottom traces:** Sample recordings of ACh-activated single-channel currents in outside-out patches excised from neurons in which type IA, IB, and II whole-cell currents could be activated by ACh. Numbered arrows show the approximate positions of the single-channel traces within a 1-s ACh pulse. Holding potential, -80 mV. **C. Top traces:** Sample recordings of ACh (1 mM)-activated IA currents in a hippocampal neuron held at +23, +3, -7, and -27 mV (top to bottom traces). Ca²⁺ concentration in the external solution was either 1 mM (●) or 10 mM (○). DHβE (0.1 μM), TTX (0.3 μM), and atropine (1 μM) were added to the external solutions. Notice that upon increasing the concentration of extracellular Ca²⁺ from 1 to 10 mM, the currents decayed faster. **Bottom graph:** ACh current-voltage relationship obtained under the two different experimental conditions. Notice that upon increasing the extracellular Ca²⁺ concentration, the reversal potential of ACh current was shifted to the right. ACh (1 mM) was applied to the neuron in a 1-s pulse every 30 s. **D.** In this model of the nAChR α subunit, the amino acid residues are numbered according to the primary structure of the *Torpedo* nAChR. Arrow A points to binding site for ACh, and B points to that for PHY.

in the sequence regions that contain elements of the ACh-binding site, but is located within the extracellular, amino-terminal region of all nAChR α subunits cloned to date, a domain suitable for the binding of extracellular ligands (Fig. 1D). Since the region bearing the PHY site is highly conserved in all the nAChR α subunits sequenced to date¹⁴, and PHY interacts with a variety of nAChR subtypes, the novel nAChR site may play a role in the control of the activation of most, if not all, nAChR subtypes. The identification of an endogenous ligand that could bind to this site and control nAChR activity would ensure the physiological relevance of this newly identified site in the process of synaptic transmission. The efficacy of PHY and PHY-like compounds as nicotinic agonists is apparently so low that although these compounds can activate single-channel currents, they are unable to evoke macroscopic currents¹³⁻¹⁵. It is possible that PHY and PHY-like compounds exert on nAChRs effects that oppose and outweigh their agonist effects, thus impairing their ability to activate whole-cell responses. Indeed, all the compounds that have been shown to bind to this novel nAChR site also have open-channel blocking properties or desensitizing effects on the nAChRs, and the concentrations at which such compounds would block or inactivate the nAChRs overlap those at which they act as agonists¹³⁻¹⁵. Nevertheless, it is tempting to speculate that ligands that would bind exclusively to the novel nAChR site could act as coagonists, thereby modulating the channel activation by ACh. Based on molecular modeling, phenanthrene-type opium alkaloids are structurally related to PHY and GAL. Interestingly, in outside-out patches excised from PC12 cells, the alkaloid codeine could activate single-channel currents apparently *via* the same mechanism as PHY. Thus, endogenous opioid-type compounds, *e.g.* endorphins/enkephalins, may be ligands for this novel nAChR site.

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NONCOMPETITIVE AGONISM AT NICOTINIC ACETYLCHOLINE RECEPTORS.

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It was a provocative finding when Albuquerque and coworkers first showed that the archetypic acetylcholine esterase (AChE) inhibitor (-)-physostigmine (Phy) could activate single-channel currents in isolated frog muscle fibers that resembled those evoked by acetylcholine (ACh), and at higher concentrations could act as an open-channel blocker (1,2). As was later found, these actions do not involve the carbamate function of Phy (3), and they are also observed with structural analogs of Phy that do not act as anticholinesterases (4). Phy and related compounds can activate nicotinic acetylcholine receptors (nAChR) from fish electrocytes (3,5,6), vertebrate muscle cells (1-3,6,7), mammalian ganglionic and neuronal tissue (4,8,9) and when ectopically expressed in fibroblasts (10) or *Xenopus* oocytes (11). Because in all of these systems nAChR activation by Phy was insensitive to established nicotinic antagonists, such as α -bungarotoxin, D-tubocurarine and methyllycaconitine (3-6,8-10), the Phy must act by binding to sites on the nAChRs that are distinct from those for ACh. Indeed, from photoaffinity labeling of *Torpedo* nAChR with tritiated Phy, proteolytic cleavage and purification of the labeled peptide, and sequence analysis, Lys-125 of the α -subunit was found to carry the label (12). From competition binding of the Phy-competitive antibodies FK1 (3) and PK1, and the ACh-competitive antibody WF6 (13) to membrane-bound *Torpedo* nAChR, the epitopes of PK1 and WF6 do not overlap, those of FK1 and WF6 partially overlap, suggesting that the binding sites of Phy and ACh are separate entities (14). This was further established by mapping the dominant prototopes of FK1 and WF6, employing as representative structural elements of the *Torpedo* nAChR α -subunit N-terminal region a panel of synthetic peptides matching in sequence preselected portions of this subunit. The dominant prototopes found in this way were the sequence regions α 118-137 for FK1 and α 181-200 for WF6 (14). Interestingly, sequence region α 118-137 displays the highest level of structural conservation in nAChR α -polypeptides, outside of the putative transmembrane domains, with more than 60% identity and an additional 29% of conservative substitutions. This evolutionary conservation is also reflected by the binding properties of antibody FK1 which has been shown by immunohistochemical and direct binding studies to bind to all nAChR preparations studied so far, including nAChRs from mammalian muscle and brain, *Torpedo* electric tissue and insect brain (14,15). From hydropathy calculations, the region surrounding α Lys-125 constitutes an amphipathic environment and may form a β -pleated sheet domain (13). Since the putative ACh binding region α 185-205 is rather hydrophilic, these general differences between the two binding domains may already significantly contribute to their ligand selectivity. Because Phy and related compounds act as nicotinic agonists, albeit *via* binding sites on the nAChR α -subunits that are separate from those for ACh and ACh-competitive ligands, they are referred to as "noncompetitive agonists" (NCA).

Following a suggestion by K.-H. Weber from Boehringer-Ingelheim, the *Amaryllidaceae* alkaloid galanthamine, a centrally acting competitive AChE inhibitor, was found to act in a similar fashion as Phy (4). Using molecular modelling approaches with galanthamine as the template, based on structural coordinates stored in the Cambridge Structural Data bank, a sizable number of structural analogs were retrieved and were submitted to electrophysiological studies with nAChR-expressing cell systems. Based on these studies, the opium alkaloid codeine also acts as noncompetitive agonist on central and peripheral nAChRs (4). From a structural comparison of the compounds identified as NCAs, a nitrogen that is cationic at physiological pH,

and that is located at an appropriate distance from a phenolic hydroxyl, appears to be essential structural elements. Although these structural properties are similar to those required for narcotic activity of exogenous phenanthrenes and endorphins, they are also found in non-narcotic drugs, such as certain dopaminergic agonists and antagonists, and some centrally acting cholinergic drugs. Interestingly, a role of cholinergic systems in the central action of narcotics has long been proposed.

Noncompetitive agonists do not activate whole-cell currents in the cellular systems tested so far, i.e. hippocampal neurons (8), muscle cells (6), *Xenopus* oocytes (11) and fibroblasts, stably expressing the chick neuronal $\alpha 4\beta 2$ nAChR (M10 cells) (10). Similarly, Phy induced only very small ion fluxes into closed membrane vesicles from *Torpedo marmorata* electric tissue (16). These and other data suggest that the physiological role of NCAs probably is modulatory rather than directly excitatory. Should there indeed exist endogenous nAChR ligands that act as "co-transmitters", they could be part of a "chemical network", overlaying the neuronal network formed by synaptic contacts.

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Comparison of native and recombinant receptor channels:
glutamate and acetylcholine receptors.

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Introduction

One of the most important problems in synaptic physiology is the identification of which receptor subtypes are responsible for mediating synaptic transmission. The advent of the methods of molecular genetics has revealed that all of the fast neurotransmitter-activated receptor-channels that mediate synaptic transmission are made up of several subunits. These studies have also shown that there are many sorts of subunits. The question is, which particular subunit combination mediates the signal in each sort of synapse? This may, of course, be an overoptimistic way of putting the question. There may not be just one answer. It is known, for example, that there is often more than one receptor type present in the same cell (and, indeed, often in the same patch of membrane). Another major problem is that most cells have not only subsynaptic receptors (those located directly under the sites of transmitter release), but also many extrasynaptic receptors (the function of which, if any, is not known). Membrane patches are almost always taken from the somatic membrane, and will almost always contain extrasynaptic receptors. If we are lucky, it may turn out that synaptic receptors are homogeneous (even though extrasynaptic receptors are not always homogeneous). In this case there will at least be a unique answer to the question that was posed above, though of course this answer will be more difficult to find if synaptic and extrasynaptic receptors differ. Recent work has shown a close similarity between somatic and dendritic glutamate receptors (Spruston, Jonas & Sakmann, 1994); this is encouraging, though the dendritic receptors are not necessarily subsynaptic.

Comparison of native and recombinant channels is one of the main methods used to elucidate the subunit composition of native receptors. The now classical studies of Mishina *et al.* (1986) showed clearly that the two forms of muscle nicotinic acetylcholine receptors (the embryonic and the adult form) were due to a switch in the subunit composition of these receptors. Since then it has been the aim of a number of groups to repeat this type of experiment in the central nervous system. Unfortunately the diversity of subunits for every known agonist-activated receptor has turned out to be much greater than could have been anticipated. Furthermore, it has become apparent that the systems used for heterologous expression of cloned receptor subunits may not always produce receptors that behave in exactly the same way as the 'same' receptors do when in their native environment. These differences appear to result, in some cases, from production of receptors from which a subunit has been omitted, but in other cases the reason for differences from native receptors remains unexplained (see Edmonds, Gibb & Colquhoun, 1994).

Studies with NMDA receptors

It has become clear that glutamate is the most important excitatory transmitter in the central nervous system. It has been suggested that the N-methyl-D-aspartate (NMDA) type of glutamate receptor is important for the understanding of a large range of physiological phenomena, though the evidence for these roles is not always unequivocal (their contribution to hyperbole in scientific writing is, however, undeniable).

It is remarkably rare for experiments from different laboratories to be done under comparable conditions. Almost always there are differences of cell-preparation, temperature, ionic composition, and so on. To cite one example, the single channel conductance is an important criterion for comparison of native and recombinant channels, but it is quite sensitive, not only to the monovalent cation concentration, but also to the extracellular calcium concentration (which varies even more from lab to lab). It was shown by Gibb & Colquhoun (1992) that the main single channel conductance level for the most common sort of NMDA receptor channel was 50 pS in 1 mM calcium, but 42 pS in 2.5 mM calcium and 66 pS in EDTA-buffered solution. Thus, to ensure comparable results, it is particularly valuable to work, in parallel, on both native and recombinant receptors in the same laboratory.

Behaviour of native NMDA-receptor channels

In order to compare the behaviour of native and recombinant receptors it is desirable to have detailed knowledge of both of them. Several publications described the details of the single channel behaviour, and especially the kinetics, of native NMDA receptors before the first receptor clones were found. This detailed information was, at the time, of limited usefulness in improving our understanding of synaptic transmission, but it has turned out to be of great value now, because it can be compared with the results obtained with different combinations of recombinant subunits.

Howe, Cull-Candy & Colquhoun (1991) described two conductance levels which could account for the large majority (more than 90%) of glutamate, aspartate, and NMDA-induced single channel openings in cultured cerebellar granule cells. The main conductance level was again about 50 pS (in 1 mM calcium), while a second 40 pS subconductance level of rarer occurrence was also found. They also analysed the number and frequency of transitions between the different conductance levels (including the shut state). Since that time many authors have used either freshly dissociated cells, or cells in brain slices, to overcome the possible hazards of culturing. Gibb and Colquhoun (1991, 1992) used both methods on hippocampal (CA1) cells. By using very low glutamate concentrations (20-100 nM), in the presence of glycine, they were able to separate individual receptor activations (see Edmonds *et al.*, 1994, for a precise definition of this term). They observed 40 and 50 pS conductance levels (in 1 mM calcium) which were very similar to those seen by Howe *et al.* (1991). They fitted shut time distributions with five exponentials, and found that the three fastest components (and probably the fourth too) were not dependent on the glutamate concentration. This allowed them to describe quantitatively the complex patterns of channel openings which can be grouped into bursts, clusters and super-clusters. It was clear that individual channel activations were often very long, and that this was one reason for the slowness of the NMDA receptor-mediated synaptic current, though concentration-jump experiments show that the latency to the first channel opening after a brief pulse of agonist may be very long, and this also contributes to the slow decay of the synaptic current (Edmonds & Colquhoun, 1992).

Recombinant NMDA-receptor channels

So far only five subunits have been found in the NMDA receptor family, NR1, which is universal, and NR2A, NR2B, NR2C and NR2D, which are more selectively distributed. The pairwise combination of NR1 with any one of the NR2 subunits gives large responses in the oocyte expression system. Stern, Béhé, Schoepfer & Colquhoun (1992) compared single channel records from several subunit pairs. In outside-out patches from *Xenopus* oocytes the combination of NR1-NR2A and NR1-NR2B gave single channels with a main conductance level of 50 pS and a subconductance level of about 40 pS which resemble closely the native receptors that have been described in cerebellar granule cells as well as the very similar channels in hippocampal neurones (Howe *et al.*, 1991; Gibb & Colquhoun, 1991, 1992, see above). This similarity was not restricted only to the two conductance levels (40 and 50 pS in 1 mM calcium), but it was found that the frequency of transitions between different conductance levels was in close quantitative agreement with the measurements of Howe *et al.* (1991) in cultured cerebellar granule cells. Furthermore the quantitative similarity extended to the distributions of open times, shut times, burst lengths etc. (Béhé *et al.* in preparation). The conclusion of this work was that low-concentration equilibrium single channel records are indistinguishable for (a) NR1-NR2A channels expressed in oocytes, (b) NR1-NR2B channels expressed in oocytes, and (c) the '50 pS' channels observed in various cultured, dissociated or brain-slice neurones. However NR1-NR2A and NR1-NR2B channels are clearly distinguishable by their different glycine sensitivities.

The NR1-NR2C subunit combination, on the other hand, gives single channels which differ in nearly all the parameters analysed, when expressed in *Xenopus* oocytes (Stern *et al.*, 1992). Their main conductance level is 36 pS with a sublevel of 19 pS which is only about half the amplitude of the main level. The development of NMDA receptors in rat cerebellar slices has recently been described by Farrant *et al.* (1994). In 14-day old rat cerebellar slices (the usual age for brain slice experiments), the channels all appeared to be of the '50 pS' type, described above. However, in older animals (in which brain slices are much harder to work with) they found that mature post-migratory granule cells contain a subtype of receptor which shows conductance levels of 33 and 20 pS in 1 mM calcium; these values are similar to those found with the NR1-NR2C combination (as expected from *in situ* hybridization studies).

The close similarity between the characteristics of native NMDA receptor single channels, and those expressed in *Xenopus* oocytes is gratifying, but it is somewhat

circular to use this observation as an answer to the question if the same channel type has the same behaviour whether it is in oocytes or in its native environment. It was, therefore, very reassuring to find that the single channel behaviour of channels produced by transient transfection of HEK 293 cells was indistinguishable from their behaviour when produced by RNA injection into oocytes (Stern, Cik, Colquhoun, & Stephenson, 1994). In this study the following single channel properties were compared: channel conductances and transition frequencies between the different levels, shut time distributions, and open time distributions conditional on the conductance level. All were similar for the two different expression systems, and this adds weight to the view that both expression systems work well for NMDA receptors,

Neuronal Nicotinic Acetylcholine receptors

The study of neuronal nicotinic receptors has proved far more difficult than that of NMDA receptors. Firstly, native nicotinic receptors are heterogeneous, even within a single membrane patch, both in sympathetic ganglia and in the medial habenular nucleus (Mathie *et al.*, 1991, Connolly *et al.*, 1995). Secondly, the amplitudes and subconductance levels are much less well-defined for native neuronal nicotinic channels than for NMDA (Mathie *et al.*, 1991). There are also serious problems in the investigation of recombinant receptors. The relative potencies of a range of agonists measured by whole-cell responses in sympathetic neurones, resemble quite closely the results measured in parallel from oocytes expressing the $\alpha 3$ - $\beta 4$ subunit combination (Covernton *et al.*, 1994; reviewed in Edmonds *et al.*, 1994). However, even muscle-type nicotinic receptors produce heterogeneous channels when expressed in oocytes (Gibb *et al.*, 1990), and similar expression of neuronal nicotinic subunits produces results that are at least as complex as for muscle type. The predominant conductance level may even depend on the particular (DNA or RNA) construct used for expression (work in progress).

The function of nicotinic receptors in the CNS is, of course, completely unknown, and, *a fortiori*, it is impossible to predict the functional consequences of the heterogeneity of the receptors seen in membrane patches. Clearly more work is needed at both the physiological and the molecular levels before much sense can be made of their widespread presence in the brain.

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*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

WEDNESDAY, NOVEMBER 9, 1994

(MORNING, SECOND SESSION)

CNS RECEPTOR FUNCTION AND STRUCTURE II

Chair: Dr. J.-P. Changeux; Co-Chair: Dr. B. Krueger

11:30	Cull-Candy, S.	Glutamate-Channels and Synaptic Transmission in Cerebellar Granular Cells
12:00	Nakanishi, S.	Molecular Diversity and Physiological Functions of Glutamate Receptors
12:30	Seeburg, P.	Glutamate-Gated Ion Channels in Brain: Properties and Genetic Control
13:00	Betz, H.	The Inhibitory Glycine Receptor: Structure, Mutants, and Postsynaptic Localization
13:30	--	Afternoon Free
15:30	--	Poster Session

GLUTAMATE-CHANNELS AND SYNAPTIC TRANSMISSION IN CEREBELLAR GRANULE CELLS

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We have used various approaches to obtain information about glutamate receptors in cerebellar granule cells, with the aim of characterizing these receptors at the single channel level and of investigating their involvement in transmission at an identified synapse (the mossy fibre input to granule cells). Although cerebellar granule cells can be readily distinguished in culture, the advent of the thin-slice technique (Edwards *et al.*, 1989) has allowed us to extend our studies to obtain high resolution patch-clamp recordings from identified cells of known developmental state, under conditions that more closely resemble those occurring *in vivo*.

Cerebellar granule cells are particularly suited to the study of synaptic currents and synaptic receptors because their electrical characteristics allow low noise whole-cell recordings (Silver *et al.*, 1992; Traynelis *et al.*, 1993). The cells have a small soma and four short unbranched dendrites. They receive, on average, only one excitatory mossy fibre input per dendrite, each with few release sites. The importance of using identified cells in our experiments has been reinforced by recent developments in the molecular cloning of glutamate receptor subunit cDNAs identifying nearly a dozen non-NMDA and five NMDA subunits. Glutamate receptors are likely to have a multimeric structure, thus the number of possible subunit combinations is very large. Because the pharmacological and biophysical properties of glutamate receptors depend critically on their subunit composition (eg Monyer *et al.*, 1992; Hollmann & Heinemann, 1994) this potential diversity has important consequences for our understanding of amino acid-mediated neurotransmission.

There are relatively few examples of identified neurons in which the types and properties of *single* NMDA and non-NMDA channels have been described. We have found that several functionally distinct non-NMDA and NMDA channel types (differing in conductances and kinetic properties) co-exist within cerebellar granule cells (Cull-Candy *et al.*, 1988; Wyllie *et al.*, 1993; Farrant *et al.*, 1994) and are attempting to determine which of these receptors are present at the synapse (Silver *et al.*, 1992; Traynelis *et al.*, 1993). In cultured neurons single-channel recordings from outside-out patches have revealed the presence of several different types of non-NMDA channels in granule cells that can be distinguished on the basis of their multiple conductance levels (Wyllie *et al.*, 1993). One type, activated both by AMPA and kainate, opens to levels of 10, 20 and 30pS. There is also a 5-10pS channel activated by AMPA, and a 1pS channel activated by kainate (seen as a noise increase). In addition to these, we have previously described the presence, in a subset of granule cells, of an unusually low conductance 'femtosiemens' channel (150fS) (Cull-Candy *et al.*, 1988). Because these low-conductance channels may be masked by higher conductance openings they have proved difficult to investigate. We have recently begun to determine the relationship between these functionally distinct responses and the expression by granule cell of different non-NMDA receptor subunits by examining recombinant non-NMDA channels.

In order to estimate the conductance of the channels underlying the non-NMDA component of the EPSC we have developed a non-stationary variance analysis method (Traynelis *et al*, 1993) that extends previous approaches (Sigworth, 1980; Robinson *et al*, 1991). When applied to the non-NMDA component of the EPSC, we obtained an estimate of ~ 20 pS for the conductance of the underlying channels (Traynelis *et al*, 1993), which corresponds well to the weighted mean conductance of the 10-30pS channels previously identified in granule cell patches (Wyllie *et al*, 1993). This has allowed us to exclude the possibility that the EPSC is carried exclusively by low conductance channels (e.g. 5-10pS, 1pS or the 'femtosiemens channels') at this synapse. Instead, most of the current appears to be carried by 10-30pS channels. In keeping with recent observations at central inhibitory synapses, a low number of channels (only 8 non-NMDA channels and 5 NMDA channels) are opened by the single transmitter packet. This contrasts with the much larger number of channels activated at peripheral cholinergic and glutamatergic nerve-muscle synapses where the transmitter packet opens, respectively, 1500 and 200 ion channels.

During early cerebellar development in the rat marked changes occur in the distribution of mRNAs encoding various NMDA-receptor subunits; NR-2A appears postnatally, NR-2B appears only transiently during the first two postnatal week, and NR-2C appears postnatally and increases to high levels in the adult (Akazawa *et al*, 1994). We have been particularly interested in the NR-2C subunit, since this is found almost exclusively in the cerebellum (Monyer *et al*, 1992; Kutsuwada *et al*, 1992). By recording from identified cells at defined developmental stages we have been able to demonstrate a corresponding change in the functional properties of NMDA receptors. NMDA receptors with single-channel properties similar to those in many central neurons (50pS main conductance) occur in premigratory and migrating granule cells (Farrant *et al*, 1994). However, more mature postmigratory cells also express an NMDA receptor with a lower single-channel conductance (20 and 33pS levels). These show distinct kinetic behaviour and correspond closely to recombinant NMDA receptors formed by coexpression of NR-1 and NR-2C subunits (Stern *et al*, 1992). Thus, it seems likely that in the cerebellum a developmentally regulated change in subunit composition may result in channels with distinct properties. It is possible that these channels could be involved in synaptic transmission in the mature cerebellum.

In young animals both evoked and miniature EPSCs have a conventional slowly rising and decaying NMDA component, which decays with a time constant of about 50ms. The fact that miniature synaptic currents, which are thought to arise from a single packet of transmitter, have both non-NMDA and NMDA components confirms that these receptors are in close juxtaposition in the postsynaptic membrane of granule cells. The resolution of our recordings in these cell is such that single NMDA channel openings can be directly resolved in the tail of the miniature currents. We have recently examined single NMDA channel openings during the tail of the evoked synaptic current, recorded when the number of activated channels was reduced to a low level with AP5. These experiments suggest that the conductance of synaptic NMDA channel may differ from channels in outside-out patches obtained from the cell soma. This was not immediately apparent since in the presence of 1mM Ca^{2+} , the single-channel conductance of NMDA receptors in outside-out patches from granule cells (and other neurons) is typically 50pS (Farrant *et al*, 1994), which appears similar to the individual postsynaptic NMDA channel

openings resolved during MEPSCs in young rats (Silver et al, 1992). However, the experiments on MEPSCs were carried out in 2mM Ca²⁺, and the conductance of NMDA channels is influenced by the external Ca²⁺ concentration raising the possibility that the conductance of the synaptic channel may approach ~60pS in 1mM Ca²⁺.

To date, our studies indicate that both synaptic and somatic NMDA channels examined in the whole-cell configuration do indeed have a conductance of 60pS in 1mM Ca²⁺, compared with a conductance of 50pS for somatic channels examined in outside-out patches under similar conditions. It would clearly be of interest to determine whether this difference in conductance is accompanied by differences in other properties of the NMDA channels. Thus our data indicate that the single channel conductance properties of synaptic and somatic NMDA channels are similar, but suggest that the channel conductance is altered by the process of forming an outside-out patch.

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MOLECULAR DIVERSITY AND PHYSIOLOGICAL FUNCTIONS OF GLUTAMATE RECEPTORS

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Glutamate receptors are essential for many integrative brain functions such as learning, memory and neural development and are also critical in some pathophysiological processes such as epilepsy and ischemic neuronal cell death (1, 2). Glutamate receptors are classified into two distinct groups of receptors termed ionotropic and metabotropic receptors. The former receptors contain glutamate-gated, cation-specific ion channel and are subdivided into NMDA receptors and AMPA/kainate receptors (1, 2). The latter receptors (mGluRs) are coupled to intracellular signal transduction through G proteins (1, 2). We have cloned and characterized the NMDA receptors and mGluRs with the aid of our functional cloning strategy that combines electrophysiology and a *Xenopus* oocyte expression systems (3-5).

Diversity of glutamate receptors

The NMDA receptors consist of two distinct types of subunits, one termed NMDAR1 and the other four termed NMDAR2A-2D (4, 6). NMDAR1 possesses all properties characteristic of the NMDA receptor-channel complex: agonist and antagonist selectivity, glycine modulation, voltage-dependent Mg^{2+} blockade, Ca^{2+} permeability and Zn^{2+} inhibition. NMDAR2A-2D show no channel activity in a homomeric structure but potentiate the NMDA receptor activity in combined expression with NMDAR1. These subunits also confer the variability in the properties of the NMDA receptors by different heteromeric subunit configurations. The NMDAR1 mRNA is ubiquitously expressed throughout the brain regions, whereas individual NMDAR2 mRNAs are distinctly distributed in different brain regions. The functional heterogeneity of the NMDA receptors in different neuronal cells are thus produced by the functional and anatomical differences of the NMDAR2 subunits. The NMDA receptor subunits probably possess an odd number of membrane-spanning domains with an extracellular domain on the N-terminal side and a cytoplasmic domain on the C-terminal side. Analysis of NMDAR1 indicated that the asparagine at the second transmembrane segment is within a channel pore and is responsible for governing a high Ca^{2+} permeability and the blockades of Mg^{2+} and other channel blockers (7).

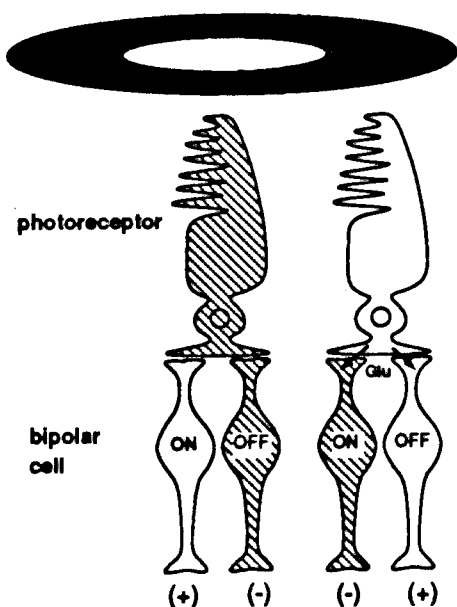
The mGluRs form a family of at least seven different subtypes termed mGluR1-mGluR7 (2, 5, 8). These receptor subtypes have seven transmembrane segments common to other members of G protein-coupled receptors but possess an unusually large extracellular domain at their amino-terminal regions and thus represent a novel family of G protein-coupled receptors. The seven mGluR subtypes differ in their agonist selectivities and signal transduction mechanisms (2). mGluR1 and mGluR5 are coupled to IP_3/Ca^{2+} signal transduction and efficiently respond to quisqualate. The other five are all linked to the inhibitory cAMP cascade, but mGluR2 and mGluR3 effectively interact with trans-1-aminocyclopentane-1,3-dicarboxylate (tACPD), whereas mGluR4, mGluR6 and mGluR7 potently react with L-2-amino-4-phosphonobutyrate (L-AP4). The seven subtypes of the mGluR family are thus classified into three subgroups according to their sequence similarities, signal transduction mechanisms and agonist selectivities. All but mGluR6 mRNA are widely but distinctly distributed in various brain regions (2). These findings strongly indicate that individual mGluR subtypes have their own functions by specializing the signal transduction and expression patterns in different nerve cells.

mGluR6 in visual transmission

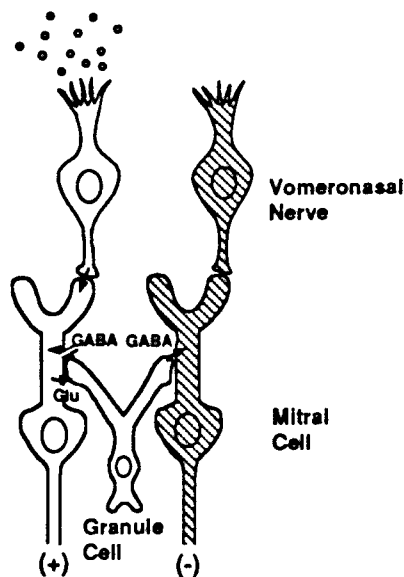
On the basis of our knowledge of the diverse members of the glutamate receptors, it is essential to explore the physiological roles of individual receptor subtypes or subunits in integrative brain functions and during neural development. We have addressed this interesting

question by several different approaches. One of the interesting systems involving a specific mGluR is visual transmission. A key processing for visual contrasts occurs at the level of bipolar cells by segregating the visual signals into ON-center and OFF-center pathways. It has been reported that synaptic transmission from photoreceptors to bipolar cells in the ON-center pathway is mediated by an L-AP4-sensitive mGluR through the coupling to the cGMP cascade. We identified mGluR6 from a retinal cDNA library and indicated that this mGluR subtype is exclusively expressed in the bipolar cell layer and selectively responds to L-AP4 after DNA transfection in CHO cells (9). Furthermore, immunocytochemical and immunoelectron-microscopic analysis provided compelling evidence that mGluR6 is restrictedly located at the postsynaptic site of the rod (ON-type) bipolar cells in the adult rat retina (10). Thus, taking together with the electrophysiological characterization of mGluR-mediated signal transduction in ON-bipolar cells, our findings introduced the following model for synaptic transmission from photoreceptors to ON-bipolar cells (Fig. 1). When light activates the photoreceptor, intracellular concentrations of cGMP decrease through the stimulation of phosphodiesterase via transducin. The decrease in cGMP concentrations hyperpolarizes photoreceptors by closing the cGMP-gated ion channel and reduces glutamate release. Under this situation, the mGluR6-G protein-phosphodiesterase system in ON-bipolar cells is inactive, and high concentrations of cGMP are maintained in ON-bipolar cells and stimulate the cGMP-gated ion channel, thus resulting in depolarization of ON-bipolar cells. Glutamate release from ON-bipolar cells is augmented and in turn excites AII amacrine cells or ganglion cells, and this excitation is transmitted to the brain. Our investigation thus indicates that a specific mGluR subtype plays a critical role in synaptic transmission in the visual system. We also demonstrated by immunocytochemical analysis that mGluR6 is initially distributed in both the cell bodies and dendrites of the bipolar cells and is gradually concentrated at the postsynaptic site during retinal development. mGluR6 in the bipolar cell thus provides the first example indicating the developmental specialization of a receptor localization in the central nervous system.

VISUAL SENSORY TRANSMISSION



OLFACTORY SENSORY TRANSMISSION



mGluR2 in olfactory transmission

We also defined the physiological role of mGluR2 in olfactory sensory transmission of the accessory olfactory bulb (AOB). In the AOB, mitral cells receive afferent inputs from the vomeronasal nerve and transmit excitatory outputs to various brain regions (Fig. 1). Granule cells are inhibitory interneurons that form typical dendrodendritic synapses with mitral cells. These synapses undergo reciprocal regulation, in which the granule cell is excited by glutamate from the mitral cell and exerts an inhibition onto the mitral cell by GABA. Our analysis with *in situ* hybridization and immunohistochemistry indicated that mGluR2 is highly expressed and localized at the dendrites of granule cells of the AOB. Because we identified 2-(dicarboxycyclopropyl)glycine (DCG-IV) as a selective and potent agonist for mGluR2, we investigated the role of mGluR2 in GABA transmission from granule cell to mitral cells by examining the effect of the DCG-IV-mediated activation of mGluR2 on GABA transmission with the use of patch-slice recording techniques. Our results indicated that glutamate released from the mitral cell activates mGluR2 at the presynaptic site of the granule cell and relieves inhibitory GABA transmission on the mitral cell (11). The granule cell forms divergent synaptic contacts with not only the original mitral cell but also a large number of neighboring mitral cells and thus causes self-inhibition and lateral inhibition via inhibitory GABA transmission. Under this synaptic network, glutamate released from excited mitral cells relieves GABA-mediated self-inhibition via the activation of mGluR2 but would maintain the lateral inhibition of unexcited neighboring mitral cells. This mechanism would evidently enhance the signal-to-noise ratio between the excited mitral cells and their neighboring mitral cells and would contribute to discrimination and resolution of different olfactory stimuli (Fig. 1).

We examined whether the proposed synaptic modulation by the activation of mGluR2 operates in olfactory sensory transmission by animal behavioral analysis. Female mice form an olfactory memory at mating, thus evoking pregnancy block after exposure to unfamiliar male pheromones of a different strain but offsetting this effect to familiar pheromones of the stud male. This olfactory block to pregnancy, known as the Bruce effect, is caused by sustained enhancement of norepinephrine in the AOB after mating. Enhanced norepinephrine persistently excites mitral cells by reducing GABA transmission from granule cells to mitral cells and results in olfactory memory formation specific to pheromones exposed after mating. Because the activation of both the norepinephrine receptor and mGluR2 reduces GABA transmission, we examined whether the activation of mGluR2 by its specific agonists could create olfactory memory formation without mating. mGluR2 agonists were infused into the AOB of females during exposure of male pheromones without mating. The females were then mated with a different strain and reexposed to pheromones (test pheromonal exposure) of the original strain. Under this protocol, the memory formation by drug infusions can be evaluated by measuring the protection of pregnancy block induced by the test pheromonal exposure. The results of this study explicitly indicated that the activation of mGluR2 induces a specific olfactory memory formation. Furthermore, this memory faithfully reflects the memory formed at mating. Thus, our study demonstrates that a specific mGluR is involved in evoking neuronal plasticity responsible for the formation of recognition memory.

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Glutamate-Gated Ion channels in Brain: Properties and Genetic Control

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In the mammalian central nervous system (CNS) L-glutamate, the principal excitatory neurotransmitter activates ionotropic receptors located in the postsynaptic membrane. These glutamate receptor channels (GluRs) are configured from different subunits of which 16 have been characterized by cloning. These can be grouped into different families based on sequences and functional properties. Native GluRs differ in biophysical and pharmacological properties and are currently classified as AMPA receptors, NMDA receptors and high-affinity kainate receptors. While the physiological role of the first two channel types is well understood, the role of the high-affinity kainate receptors in central neurons is not resolved. All three receptor types have been implicated in mediating neuronal death in pathophysiological conditions such as epilepsy, head trauma, ischemia and hypoxia.

AMPA receptors mediate the majority of the fast synaptic excitatory action of L-glutamate. These channels possess rapid onset, offset and desensitization kinetics, and are largely impermeable to Ca^{2+} ions. AMPA receptors assemble from subsets of four subunits, GluR-A to -D (alternatively termed GluR1 to 4) which share approximately 70% sequence identity. Each subunit when expressed in heterologous expression systems assembles with itself to form homooligomeric channels that can be activated by L-glutamate and other excitatory amino acid analogs (kainate, domoate). However, when several AMPA receptor subunits are co-expressed, heterooligomeric channels form with properties distinct from those of homooligomeric channels. One particular property that varies with subunit composition is the Ca^{2+} permeability of AMPA receptor channels. This divalent ion permeability is very low for channels configured with participation of GluR-B but is high for AMPA channels formed in the absence of GluR-B. Since GluR-B is highly expressed in most CNS structures, native AMPA receptor channels have generally low Ca^{2+} permeabilities. This predicts that neurons can regulate the Ca^{2+} influx through AMPA receptors by controlling the read-out of the GluR-B gene. Indeed, molecular and biophysical analysis of single live neurons in brain slices shows that cells with Ca^{2+} permeable AMPA receptors (generally GABA-ergic interneurons) express little GluR-B relative to other subunits whereas neurons with low Ca^{2+} permeability of AMPA receptors have

high levels of GluR-B relative to other AMPA receptor subunits. Thus, the Ca^{2+} permeability of AMPA receptors depends on the fractional GluR-B content. It appears that at relatively low GluR-B levels, two AMPA receptor populations form; those incorporating GluR-B which have low Ca^{2+} permeability, and those formed without GluR-B which are characterized by high Ca^{2+} permeability. Mutational analysis of GluR-B revealed that the determinant for the dominant behaviour of this subunit regarding divalent ion permeability of AMPA receptor channels resides in the channel forming region M2. GluR-B carries a positively charged residue (arginine, R) in M2 while all other AMPA receptor subunits carry an uncharged residue (glutamine, Q) in the homologous position. Thus, the particular residue in the Q/R site of M2 profoundly influences the ion conductance and gating properties of AMPA receptor channels. However, the arginine residue of GluR-B M2 is not encoded by the GluR-B gene whose sequence specifies that a glutamine residue should occupy the Q/R site. It appears that a single nucleotide in the CAG codon for glutamine is changed at the GluR-B transcript level to generate the CGG codon for the critical arginine determinant in GluR-B. This process termed RNA editing is mediated by an intronic sequence in the GluR-B gene, which is unique to that gene and is not found in the genes for the other AMPA receptor subunits. This explains why the Q to R switch occurs only for GluR-B. However, it has been demonstrated that RNA editing also affects the Q/R position in several subunits of high-affinity kainate receptors in central and peripheral neurons.

We have recently found another position in AMPA receptors where RNA editing changes a gene-specified codon. The position is in an extracellular receptor domain, adjacent to the alternatively spliced modules Flip and Flop. In this position, a gene-directed arginine (R) codon is converted into a glycine (G) codon in GluR-B, -C, -D mRNA but not in GluR-A. Interestingly, the extent of editing at this R/G site is developmentally controlled. Furthermore, AMPA receptor channels edited in the R/G position have faster recovery rates from L-glutamate-induced desensitization. This suggests that RNA editing may be involved in the fine-tuning of excitatory transmission.

Central excitatory synapses often operate with both, AMPA receptors and NMDA receptors. The latter possess high Ca^{2+} / Na^{+} permeability ratios, but are characterized by slower kinetics than AMPA receptor channels. Furthermore, NMDA receptor activity is voltage-dependent due to a voltage-dependent Mg^{2+} block of the ion pore. An additional difference to AMPA receptors is that NMDA receptor activation requires the presence of glycine as co-agonist to L-glutamate. In molecular terms,

NMDA receptors are configured from two different subunit types termed NMDAR1 (NR1) and NMDAR2 (NR2). For the latter subunit there exist four variants (NR2A to D) each encoded by its own gene. The NR1 subunit gene can be alternatively spliced to yield 8 different molecular variants. Thus, central neurons can carry different NMDA receptors which also differ in biophysical and pharmacological properties. The NR2 subunit genes show strict developmental and regional expression patterns. The participation of NR2 subunits in NMDA receptor formation endows NMDA receptors with subtype-specific properties, e.g., strength of Mg^{2+} block, Ca^{2+} block of Na^+ current, desensitization time course, time course of deactivation, and glycine sensitivity. Interestingly, the hallmark properties of NMDA receptors, high Ca^{2+} permeability and voltage-dependent Mg^{2+} block were traced to an asparagine residue in the M2 of its subunits. The critical asparagine (N) occupies a position homologous to the Q/R site of AMPA receptor subunits. Indeed, the substitution of Q or R for N in AMPA receptors endows them with high Ca^{2+} permeability.

NMDA receptors have been implicated as key players in paradigms of neuronal plasticity and in ischemic cell death. Understanding the molecular composition and regulation of NMDA receptors is therefore of considerable interest for neuroscientists and neurologists alike. The complex receptor structure possesses several allosteric sites through which receptor activation can be modulated. One such site is termed the redox site at which reducing agents can potentiate glutamate-activated NMDA currents. This potentiation is thought to play a role in ischemic and traumatic injury and leads to excessive influx of Ca^{2+} into the channel-carrying cell. It can be shown that the time course and extent of potentiation is subunit specific and is correlated with differences in kinetic parameters.

THE INHIBITORY GLYCINE RECEPTOR: STRUCTURE, MUTANTS AND POSTSYNAPTIC LOCALIZATION.

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The postsynaptic glycine receptor (GlyR) is a heteromeric ligand-gated chloride channel protein, which mediates inhibition in spinal cord and other regions of the central nervous system (1). Using protein microsequencing and homology screening techniques we have isolated cDNAs and genomic DNAs encoding various subunits of this receptor system. Expression of cloned ligand binding α subunits in *Xenopus* oocytes or mammalian cell lines creates glycine-gated strychnine-sensitive chloride channels, which mimic receptors in primary spinal neurons in most of their pharmacological properties. Co-expression of the structural β subunit modifies the elementary conductance and blocker sensitivity of the GlyR chloride channel. Site-direct mutagenesis experiments indicate that this is due to amino acid substitutions within the channel forming M2 segment of the β subunit (2).

Using recombinant expression and site-directed mutagenesis we have recently identified several amino acid determinants of ligand binding to the α subunits of the glycine receptor. Our data suggest that the agonist (and antagonist) binding site of the GlyR is formed from at least three distinct regions of the extracellular domain (3). A central F-X-Y motif is crucial for agonist selectivity (4), and also conserved in the glycine binding site of the NMDA subtype of glutamate receptors (5).

Different hereditary neurological diseases are known to affect GlyR function. Typically the affected animals and patients show startle reaction, myoclonus and other convulsive symptoms which in severe cases affect the life span of the affected individuals. The dominant hyperekplexia mutation in man causes a reduction in GlyR agonist

affinity and chloride conductance and results from a point mutation in the $\alpha 1$ subunit of the adult GlyR isoform in spinal cord (6). Similarly, the recessive mouse mutant spasmodic shows a significant reduction in agonist affinity, which again is due to a single nucleotide exchange in the $\alpha 1$ subunit gene (7). The mouse mutant spastic in contrast displays a recessive phenotype, which becomes apparent only two weeks after birth and results from the insertion of a line L1 element, which causes aberrant splicing of β subunit transcripts (8). Apparently, all the presently known neurological mutations causing startle symptoms affect either the agonist affinity or the assembly of the GlyR.

The mechanism of GlyR assembly has been investigated by electrophysiological methods combined with site-directed mutagenesis (9). Expression of a low affinity mutant of the $\alpha 2$ subunit with the $\alpha 1$ and β subunits indicates that GlyRs assembled from $\alpha 1$ and $\alpha 2$ polypeptides contain variable subunit ratios, whereas $\alpha \beta$ heterooligomers have an invariant 3:2 stoichiometry. Analysis of different α/β chimeric constructs revealed that this difference in assembly behaviour is mediated by the N-terminal extracellular regions of the receptor subunits. Substitution of residues diverging between the α and β subunits identified combinations of sequence motifs ("assembly boxes"), which are essential for proper subunit stoichiometry.

Upon affinity chromatography of the GlyR, a 93 kDa polypeptide consistently co-purifies with the pentameric receptor complex. This protein, gephyrin, is homologous to molybdopterin biosynthesis genes in *E. coli* and *Drosophila* and exists in several splice variants in the mammalian brain. Immunoelectron microscopy revealed that gephyrin co-localizes with the GlyR at glycinergic postsynaptic membrane specializations. *In vitro*, gephyrin binds with high affinity to polymerized tubulin. Recent immunocytochemical and *in situ* hybridization studies show that gephyrin is also found at other than glycinergic synapses. This protein therefore may have a more general role in the organization of postsynaptic neurotransmitter receptors. Antisense inhibition of gephyrin expression prevents the postsynaptic

localization of glycine receptors in cultured spinal neurons (10). Biochemical data suggest that this postsynaptic localization process involves direct interactions of gephyrin with both receptor polypeptides and polymerized subsynaptic tubulin, e.g. the cytoskeletal apparatus.

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*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

THURSDAY, NOVEMBER 9, 1994

(MORNING, FIRST SESSION)

PROGRESS REPORTS BY SELECTED SPEAKERS

Chair: Dr. T. Narahashi; Co-Chair: Dr. W. Van der Kloot

- | | | |
|-------|------------------|---|
| 8:00 | Froehner, S. | The Submembrane Machinery for Acetylcholine Receptor Clustering |
| 8:20 | Tzartos, S.J. | Acetylcholine Receptor Tyrosine and Serine Phosphorylation, Monoclonal Antibodies as Site-Specific Tools for Phosphorylation and Channel Function |
| 8:40 | Fuchs, S. | Mapping of Functional Sites at the Nicotinic Acetylcholine Receptor |
| 9:00 | Conti-Fine, B.M. | A "Neuronal" Nicotinic Acetylcholine Receptor Regulating Cell Adhesion is Expressed in Human Epidermal Keratinocytes |
| 9:20 | Wonnacott, S. | Presynaptic Nicotinic Receptor Modulation of Dopamine Release in Rat Brain |
| 9:40 | Arneric, S. | Cholinergic Channel Activators (ChCAs) for the Potential Treatment of Alzheimer's Disease |
| 10:00 | Doctor, B.P. | Modulation of Catalysis and Inhibition of Acetylcholinesterase by Monoclonal Antibodies |
| 10:20 | -- | Coffee Break -- Poster Session |

THE SUBMEMBRANE MACHINERY FOR ACETYLCHOLINE RECEPTOR CLUSTERING

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Clustering of nicotinic acetylcholine receptors (AChRs) is one of the earliest signs of membrane differentiation at newly-formed neuromuscular synapses. Small aggregates of AChR begin to form within hours of the initial contact between nerve and muscle, and continue to grow in size and density during prenatal and postnatal development. At the adult rat neuromuscular junction, AChR are present at densities of 8000-10,000 receptors per μ^2 of postsynaptic membrane surface. Since AChR are transmembrane proteins that are inherently capable of diffusion within the plane of the membrane, they must be anchored at synaptic sites, presumably via interactions with the postsynaptic cytoskeleton and other proteins. Signals from the nerve that regulate AChR clustering, such as agrin, may act on the cytoskeleton to stimulate its assembly or its interaction with the receptor (Froehner, 1993).

The 43K protein, a peripheral membrane protein associated with cytoplasmic sites on the AChR, appears to be a key element of this complex. Recombinant AChRs expressed in oocytes or fibroblasts are diffusely-distributed on the cell surface. When coexpressed with the 43K protein, AChRs become organized into clusters. Clustering activity is inherent to the 43K protein, since clusters of 43K protein form on the membrane in the absence of the AChR (Froehner et al., 1990; Phillips et al., 1991). The molecular mechanisms by which these clusters form is unknown, but may involve other proteins endogenous to the oocyte membrane. Alternatively, 43K proteins may be capable of lattice formation. Two conserved structural motifs, a cysteine-rich, zinc finger-like sequence near the carboxy terminus, and a potential leucine zipper, may be important in 43K protein clustering. We have shown by expression of fusion proteins that the cysteine-rich sequence of 43K protein binds zinc in a specific manner (Scotland et al., 1993). Furthermore, mutation of two histidine residues located at strategic sites in the zinc finger greatly reduces both zinc binding and the ability of the 43K protein to form clusters. Thus, the zinc finger may be an important feature that mediates interactions with other postsynaptic proteins. Studies of the leucine zipper motif are in progress.

In addition to the 43K protein, several other postsynaptic cytoskeletal proteins may play an important role in AChR clustering. These include actin, spectrin, and certain members of the dystrophin family, including utrophin and the 87K protein. The dystrophin family and the proteins associated with them have recently taken on added importance with the finding that two proteins of this complex (α - and β -dystroglycan) comprise a potentially

important agrin binding site (reviewed in Sealock and Froehner, 1994). α -dystroglycan is an extracellular, heavily-glycosylated protein that binds both laminin and agrin (Ervasti and Campbell, 1993). It is associated through the transmembrane β -dystroglycan with dystrophin/utrophin. Whether α -dystroglycan is the functional agrin binding protein is uncertain, however.

Another protein associated with the dystrophin family of proteins is syntrophin (also known as 58K protein and DAP-59). Syntrophin was first identified in Torpedo postsynaptic membranes as a dystrophin-associated protein (Butler et al., 1992). Subsequent studies showed that syntrophin is associated with all members of the dystrophin family, including utrophin, and the short forms (71K, 87K) which contain only the cysteine-rich, carboxy terminal (CRCT) superdomain (Kramarcy et al., 1994). CRCT is also the site of association between dystrophin/utrophin and β -dystroglycan. Three isoforms of syntrophin with extensive sequence homology have been identified by molecular cloning (Adams et al., 1993; Ahn et al., 1994). These can be divided into two groups, based on their isoelectric points: α -syntrophins (acidic) and β -syntrophins (basic). Two forms of β -syntrophin (β 1 and β 2) were found. α 1-syntrophin is expressed at highest levels in skeletal muscle. Both β -syntrophins have a broad tissue expression pattern, although the levels in skeletal muscle are low. The expression patterns of α and β -syntrophins are similar to dystrophin and utrophin, respectively.

To identify the form(s) of syntrophin associated with the postsynaptic membrane, we prepared isoform-specific antibodies to α 1 and β 2 syntrophin and determined their localizations in skeletal muscle (Peters et al., 1994). α 1 syntrophin is found at the neuromuscular synapse but is also associated with the general sarcolemma, i. e., its distribution is indistinguishable from dystrophin. In mdx muscle, which lacks dystrophin, α 1 syntrophin staining of the sarcolemma is dramatically reduced, although not entirely eliminated. Thus, in addition to dystrophin, α 1-syntrophin may also be associated with another dystrophin-like protein, possibly the 87K protein.

Immunostaining for β 2-syntrophin is localized to the neuromuscular junction; no extrasynaptic staining was found. Thus, at this synapse, β 2-syntrophin may be associated with utrophin. Further study by biochemical techniques is required to confirm this association, however. The localization of β 1 syntrophin has not yet been determined.

Much of our knowledge of cytoskeletal and extracellular postsynaptic proteins can be brought together in a model in which α - and β -dystroglycan link agrin binding to the cytoskeleton. Several important questions remain to be answered, however. What is the nature of association between AChR/43K protein and utrophin complex? This may occur by direct interaction between the 43K protein and utrophin, or possibly indirectly via actin, or other proteins. Since α -dystroglycan is distributed over the entire

muscle cell membrane, how does agrin come to be localized at the synapse? One possibility is that synaptic and extrasynaptic α -dystroglycan are structurally (and functionally) different, with the junctional form preferentially binding agrin (instead of laminin). Alternatively, the associated proteins (utrophin, β 2 syntrophin) may indirectly alter the binding preference of α -dystroglycan. And finally, if α -dystroglycan is the functional agrin receptor, what is the mechanism by which it induces clustering of AChR?

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ACETYLCHOLINE RECEPTOR TYROSINE AND SERINE PHOSPHORYLATION. MONOCLONAL ANTIBODIES AS SITE-SPECIFIC TOOLS FOR PHOSPHORYLATION AND CHANNEL FUNCTION.

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Phosphorylation of the nicotinic acetylcholine receptor (AChR) has been implicated in the assembly, clustering, regulation of function and degradation rate of the molecule. The AChR is phosphorylated on at least eight residues by a cAMP-dependent protein kinase, protein kinase C and protein tyrosine kinases. The cAMP-dependent protein kinase(s) phosphorylates residues γ Ser353, δ Ser361 and δ Ser362, protein kinase C phosphorylates δ Ser361 and, presumably, α Ser333, while protein tyrosine kinases phosphorylate residues β Tyr355, γ Tyr365 and δ Tyr372. Thus, all the identified AChR phosphorylation sites are in the major cytoplasmic domain of each subunit, between transmembrane segments M3 and M4. The availability of specific probes for each of these phosphorylation sites should prove invaluable in the study of the structure and activity of the AChR.

Monoclonal antibodies (mAbs) against specific sites on the AChR constitute a powerful set of probes for the study of AChR localization, characterization, structure, function, metabolism and pathogenicity (reviewed in ref. 1). Extensive epitope characterization of many of our 150 anti-AChR mAbs has rendered them valuable in many studies. Immunizations of rats with intact AChRs resulted in the production of mainly mAbs directed against the extracellular Main Immunogenic Region (MIR), located on and around α 67-71 (1,3). This site is not involved in channel function mechanisms. Overall, only four out of sixty tested mAbs of ours have been found to inhibit the carbamoylcholine induced opening of the ion channel. Two of these mAbs were efficient blockers whereas the other two were weak blockers (2). These four mAbs seem to bind to the cytoplasmic side of the AChR.

Many mAbs against the cytoplasmic parts of all AChR subunits were earlier produced by injecting rats with SDS-denatured AChR or its purified subunits (see ref. 1). Recently we have mapped in detail the exact epitopes of these mAbs by the use of several hundreds of all possible overlapping peptides of various sizes. The peptides were synthesized and remained attached on polyethylene rods according to the Pepscan technique of Geysen. mAb binding was tested by ELISA. The exact limits of the epitopes for 45 such mAbs were localized; four- to eleven-residue long epitopes were identified. In addition, for certain of these epitopes, the binding role of each individual amino acid residue was evaluated by the use of single-residue peptide analogues. These peptide binding results correlated well with several other epitope mapping approaches (including antibody-competition, electron microscopy, species cross-reactivity and mapping by proteolytic polypeptides), proving the validity of both the present as well as the earlier approaches. Two additional mAbs could be indirectly mapped by combining data from different approaches. The molecular basis of the cross-reactive character of several mAbs was also elucidated from their binding to conserved or phylogenetically altered sequences. Perhaps most importantly, these studies revealed interesting

information on the putative role of AChR phosphorylation and made available valuable probes for AChR phosphorylation studies.

Below are described some of the data obtained by mapping the epitopes for mAbs to α -, β -, γ - and δ -subunits, placing emphasis on the relationship between AChR phosphorylation and channel function.

mAbs to the α -subunit: Fourteen anti-AChR mAbs were mapped to cytoplasmic epitopes (4,5). Two of these mAbs bound to epitopes which may include the single α -subunit phosphorylation site α Ser333. One of the latter (mAb 13) is the only anti- α -subunit mAb of our library known to partially inhibit channel opening.

mAbs to the β -subunit: The epitopes for 15 mAbs were mapped. Eleven mAbs bound to a Very Immunogenic Cytoplasmic Epitope on the β -subunit (VICE- β) at β 352-359, which contains the phosphorylation site β Tyr355, and to the corresponding sequence of human AChR. The contribution of each VICE- β residue to mAb binding was then studied using peptide analogues with single amino acid substitutions. Most residues, including β Tyr355, proved critical for mAb binding. Two of our four mAbs known to block the ion channel were found to bind at (the efficient blocker mAb148), or close to (the weak blocker mAb10) VICE- β . Tyrosine phosphorylation of the *Torpedo* AChR by endogenous kinases selectively reduced the binding of some VICE- β mAbs, including mAb148 (6).

mAbs to the γ -subunit: The epitopes for five anti- γ -subunit mAbs were precisely mapped. Those for three mAbs were found to include the single γ -subunit phosphorylation site γ Tyr365. The mAb-binding role of γ Tyr365 within these epitopes was then tested by the use of peptide analogues with single amino acid substitutions. The presence of γ Tyr365 proved critical for the binding of some of the corresponding mAbs. Finally, tyrosine phosphorylation of the *Torpedo* AChR by endogenous kinases selectively reduced the binding of the above three mAbs. One of these mAbs is the second known efficient blocker of AChR channel function.

mAbs to the δ -subunit: The epitopes for eleven anti- δ -subunit mAbs were precisely localized by the sequential use of three series of peptides: decapentapeptides overlapping by ten residues, decapeptides overlapping by nine residues and, finally, peptides of continuously smaller size for characterizing the precise epitope limits. Five mAbs bound between M4 and the C-terminal end, i.e. to a presumably extracellular segment. These mAbs do not bind at the membrane embedded AChR, probably due to proximity of their epitopes to the plasma membrane. Interestingly, when bound on the Triton X-100-solubilized AChR they inhibited binding of mAbs directed to cytoplasmic sites on γ - and δ -subunits; this suggests either an allosteric interaction, or that the C-terminal in the solubilized AChR is translocated and approaches cytoplasmic regions.

Four anti- δ -subunit mAbs bound within the region δ 350-380, which contains all of the δ -subunit phosphorylation sites (δ Ser361, δ Ser362, δ Tyr372 and δ Ser377). Specifically, the epitope for one mAb (δ 365-375) contains δ Tyr372, the epitope for another two mAbs (δ 376-381) contains δ Ser377, and the epitope for a fourth mAb (δ 350-359) is close to Ser361 and Ser362 and includes parts of the corresponding phosphorylation consensus sequences. Using peptide analogues with single residue substitutions, δ Tyr372 was found to be essential for the binding of the corresponding mAb, while tyrosine phosphorylation of *Torpedo* AChR selectively inhibited its binding to the intact AChR.

Overall, concerning the relation between AChR phosphorylation and channel function, all four mAbs of ours known to affect the opening of the AChR channel seem to be related with phosphorylation sites of the AChR. The correlation is most remarkable for the two best mAb blockers which bind on the tyrosine phosphorylation sites of the β - and γ -subunits and their binding to the AChR is inhibited by the phosphorylation on the corresponding sites. We conclude that most phosphorylation sites of the AChR are located on a cytoplasmic "ring" that cytoplasmically surrounds and controls the ion channel.

The above data and the availability of the defined mAb probes should facilitate the study of the functional role of single AChR phosphorylation sites.

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MAPPING OF FUNCTIONAL SITES AT THE NICOTINIC ACETYLCHOLINE RECEPTOR

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The nicotinic acetylcholine receptor (AChR) is an oligomeric protein composed of four subunits present in a stoichiometry of $\alpha_2\beta\gamma\delta$. The α -subunit contains the cholinergic binding site and is also the target for most autoantibodies in myasthenia gravis. In the following we will report on studies from our laboratory for mapping functional sites on AChR. Specifically we will concentrate on: (i) The ligand-binding site of AChR and (ii) Identification of AChR epitopes by a phage-epitope library.

The binding site of AChR from animals resistant to α -neurotoxins

The ligand binding site of AChR is located in the α -subunit, within a small fragment containing the tandem cysteines at positions 192 and 193. Previously, we have demonstrated that a synthetic dodecapeptide from the α -subunit, corresponding to amino acid residues 185-196, and containing the two tandem cysteines, contains the essential elements for ligand binding (Neumann et al., 1986). In an attempt to elucidate the molecular basis for the ligand binding site of AChR we chose to study non-classical muscle AChR from animals that are resistant to α -neurotoxins and whose receptors do not bind α -bungarotoxin (α -BTX). We analyzed the binding site of AChR from three different snakes, mongoose and several other animal species that exhibit varying degree of resistance to α -BTX (Neumann et al., 1989; Barchan et al., 1992; Kachalsky et al., 1993). The domain of the α -subunit (amino acid residues 122-205), which contains the four extracellular cysteines (128,142,192,193) was amplified by RT-PCR, cloned, sequenced and expressed in *E. coli*. Most substitutions are present in the presumed ligand-binding site in the vicinity of cysteines 192 and 193. In the snakes five major substitutions occur within amino acid residues 184-194, at positions 184 (Trp to Phe), 185 (Lys to Trp), 187 (Trp to Ser), 194 (Pro to Leu) and 189. This latter position which is an aromatic residue in animals sensitive to α -BTX is an Asn in the snake and form a putative N-glycosylation site.

As some of the sequence differences in the snake AChR could be species-specific, we extended our study to a mammal, mongoose, which is resistant to α -neurotoxins and includes snakes in its diet. Cloning and sequence analysis of the mongoose AChR α -subunit fragment (residues 122-205) revealed high homology with the respective mouse fragment. Five of the seven amino acid differences between the mouse and the mongoose fragments concentrate in the vicinity of the tandem cysteines in a stretch of eleven amino acid residues. Four of these differences are at positions 187 (Trp to Asn), 189 (Phe to Thr), 194 (Pro to Leu) and 197 (Pro to His), which are conserved in animal species that are susceptible to α -BTX, and in three (187, 189 and 194) there are major substitutions also in the snake. The mongoose AChR has also a putative glycosylation site (Asn 187) in the ligand binding site. Thus, glycosylation within the binding site may represent an additional evolutionary means for protection against peptide neurotoxins.

Recently, we have cloned by RT-PCR the entire mongoose AChR α -subunit gene (Asher and Fuchs). Alignment of the mouse and mongoose sequences shows 89% homology in the nucleotides and 93% homology in the deduced amino acids. There are 29 amino acid differences between the mouse and the mongoose α -subunits, only six of which are at positions that are conserved in all animal species that bind α -BTX, and four of these six are the ones in the binding site domain.

In addition to the snake and mongoose AChR, we have cloned, sequenced and expressed the ligand binding domain from the hedgehog, shrew and cat (Barchan and Fuchs). The hedgehog fragment did not bind α -BTX, and similarly to the snake and mongoose, it had substitutions at the conserved positions 187 and 189. This verifies that changes at these positions from aromatic residues to non aromatic ones, may be sufficient for conferring toxin resistance. Nevertheless, it should be noted that the human AChR fragment, which binds α -BTX, though to a lesser extent than the mouse, cat and shrew fragments, also has substitutions at the positions 187 and 189 from aromatic to non aromatic residues. Thus, the nature of the substitutions at the binding site, and possibly changes in other positions which may affect the charge and/or conformation of the binding site, also contribute to determining α -BTX resistance.

To further identify the amino acids participating in ligand binding, a series of point mutations were performed, changing residues in the mongoose fragment to those in the mouse sequence (Kachalsky et al., 1993). Mutations were created, each alone or in combination at positions 187, 189, 194 and 197. Analysis of the binding of the various mutated and expressed fragments (residues 122-205) to α -BTX, indicated that only the fragment in which all four positions were mutated to the mouse residues, exhibited α -BTX activity, similar to that of the mouse fragment. One double mutation at positions 194 and 197, mutating the mongoose residues to prolines, resulted in a fragment which bound α -BTX, to about 50% of the binding of the mouse fragment. These two prolines which are present in all toxin-sensitive species appear to be important in forming the required conformation for toxin-binding. All other mutations of one, two or even three of these residues to the ones present in the mouse AChR resulted at most in just a small increase in toxin binding. Our studies indicate that changes in certain amino acid residues in the binding site domain, are crucial in creating the appropriate structure that determines α -BTX resistance, and that this resistance may be achieved by various combinations of these residues.

Identification of epitopes in AChR by a phage-epitope library.

Epitope libraries are random libraries of large collections of peptides which can be constructed by chemical or molecular biological techniques. The latter are phage-epitope libraries in which each peptide is displayed on the surface of a bacteriophage particle and is encoded by a randomly mutated region of the phage genome, thus associating each unique peptide with the DNA molecule encoding it. We employed a phage-epitope library to identify epitopes for several anti-AChR monoclonal antibodies. We have applied the phage library to select a protein-derived epitope which represents a conformation-dependent determinant in AChR (Balass et al., 1993). Synthetic peptides corresponding to sequences of the original protein may not be suitable for the identification of such structural determinants. A phage epitope library was employed to select a hexapeptide mimotope to an anti-site monoclonal antibody (mAb 5.5). This mAb binds to muscle AChR of different species, inhibits the

binding of α -BTX to these AChRs, accelerates the degradation of AChR, blocks the carbamylcholine-induced Na^+ influx to muscle cell cultures, and induces a neuromuscular block in hatch chickens. We identified a hexapeptide which interacts specifically with mAb 5.5 and mimics its natural conformation-dependent epitope, both in vitro and in vivo. The affinity purified phage clones carried the sequence DLVWLL, and the synthetic peptide DLVWLL inhibited the binding of mAb 5.5 to the peptide-presenting phage and to AChR. Bioactivity of the peptide mimotope was demonstrated by inhibiting the effect of mAb 5.5 in hatch chickens. The myasthenia-like symptoms induced in chickens by passive transfer of mAb 5.5 were specifically abolished by the peptide. By extending the synthetic hexapeptide mimotope selected by mAb 5.5, on both ends with 4-5 amino acid residues, we got peptides that were one to two orders of magnitude more potent than the hexapeptides, in their interaction with the antibodies. Thus, peptide epitopes selected from phage-epitope libraries may be applied for drug design, for in vivo blocking of immunopathological antibodies in myasthenia and for the development of diagnostic markers.

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A "NEURONAL" NICOTINIC ACETYLCHOLINE RECEPTOR REGULATING CELL ADHESION IS EXPRESSED IN HUMAN EPIDERMAL KERATINOCYTES

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Several non-excitabile cells synthesize, release, and degrade the neurotransmitter acetylcholine (ACh) (1 and references therein). The function(s) of ACh in non-excitabile cells, and the receptor(s) mediating them are unknown. Also, neurons express abundant nicotinic acetylcholine receptors (AChRs) outside the synapses (e.g. see ref. 2), whose function(s) is(are) also unknown. The presence of ACh and its receptors at locations not related to synaptic activity raises the possibility that ACh may have receptor-mediated cellular functions other than those related to transmission of nerve signals.

Keratinocytes (KC) are skin epithelial cells which, like neurons, originate from the ectoderm, and can process several neurotransmitter (1 and references therein). Human KC both *in vivo* and *in vitro* synthesize, store, secrete and degrade ACh (1). Since these cells can be easily obtained and readily cultivated, they provide a useful *in vitro* system to investigate the function of cholinergic systems in non-excitabile cells.

In the present study we investigated the presence, function, and structure of nicotinic AChR(s) in human KC.

We first investigated if human KC express functional AChRs. The presence, ion gating, and pharmacological properties of AChRs in human cultured human KC were investigated in patch-clamp experiments. Single-channel currents activated by ACh were recorded from outside-out patches in the presence of atropine (1 μ M). The predominant conductance state activated by ACh was 32 pS i.e. the same as that of the most abundant AChRs in the neurons of sympathetic ganglia, which contain the α 3 subunit in association with the β 4 and the α 5 subunits or the β 2 subunit (3,4).

Single-channel currents with the same properties as those activated by ACh were elicited by (+)-anatoxin-a, a specific and selective nicotinic agonist (5). The channels activated by ACh were blocked by Mecamylamine (1 μ M) and κ -bungarotoxin (κ -BTX) (10 nM). The effect of Mecamylamine was reversible after removal of the drug; that of κ -BTX was irreversible, in agreement with the demonstrated pharmacological properties of these nicotinic antagonists on ganglionic AChRs and, for κ -BTX, also on AChRs expressed in oocytes and formed by the α 3 or the α 4 subunits (6).

The level of expression was investigated by binding of radiolabelled κ -BTX, ¹²⁵I- κ -BTX bound to ~6,000 sites/cell.

To directly ascertain the subunit composition of the KC AChR revealed by the patch clamp experiments, we looked for expression in human KC of mRNA encoding neuronal AChR α and β subunits which have shown or proposed to be part of ganglionic AChR complexes, since they have pharmacological and ion gating properties similar to those found for KC AChRs.

Research (to B.M.C.-T.), a research grant from the Dermatology Foundation (to S.A.G.), and by NIH Grants NS 25296 and ES 05720 and NIMH Center for Neuroscience and Schizophrenia (P-50MH44211) (to E.X.Albuquerque). R.M.H. is the recipient of the Robert G. Sampson Neuromuscular Disease Research Fellowship from the Muscular Dystrophy Association.

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Expression of AChR subunits was investigated by PCR using primers specific for different known AChR subunits. Primers matching the $\alpha 3$, $\beta 2$ and $\beta 4$ human sequences consistently amplified products of the expected size from cDNAs of mature human KC isolated from neonatal foreskins. Similar results were obtained consistently in cultured human KC for the $\alpha 3$ and the $\beta 4$ subunits, while the $\beta 2$ product was detected sporadically as a faint band. It is therefore possible that expression of the $\beta 2$ subunit may be affected by the culture conditions, as compared to the physiological conditions in the skin.

To verify that the appropriately sized bands resulting from PCR amplification of human KC transcripts using the $\alpha 3$, $\beta 2$ and $\beta 4$ primers did in fact represent "neuronal" AChR subunits, we cloned and sequenced the PCR products. The sequences obtained confirmed that they are true $\alpha 3$, $\beta 2$ and $\beta 4$ AChR subunits.

The subunit composition of the AChR expressed by human KC was investigated by immunofluorescence of sections of normal human skin, or KC grown on glass cover slips, using monoclonal and polyclonal antibodies against each subunit of mammalian muscle AChR (α , β , γ , δ , and ϵ), and polyclonal antibodies against different rodent neuronal α and β subunits ($\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\beta 2$), raised in rabbits injected with pools of synthetic non-conserved sequence regions of one subunit. We did not have antibodies against the $\beta 4$ subunit. However, the anti- $\beta 2$ antiserum recognized primarily an epitope within a sequence region which was highly conserved in the $\beta 4$ subunit. It should be noted that the $\beta 2$ and the $\beta 4$ subunits have a high degree of overall identity (~64%, see ref. 7). Therefore antibodies against this sequence might not discriminate between $\beta 2$ and $\beta 4$ subunits.

Only anti- $\alpha 3$ and $\beta 2$ antisera stained KC, both in skin sections and in cell cultures. The staining was abolished by pre-incubating the serum with the peptides used for the immunization. In the epidermis, the middle and upper layers were brightly stained. They are formed by spinous and granular KC, which are the most mobile epidermal cells, as they migrate from the basal layer toward the skin surface. In cultured KC, anti- $\alpha 3$ and - $\beta 2$ antibodies produced an intercellular staining pattern consistent with binding to a membrane-associated protein.

We started investigating possible cellular functions mediated by this receptor. Since previous experiments indicated that ACh can modify the motility of cultured KC, we first investigated whether this and related cell functions could be mediated by nicotinic receptors. We used assays of cell adhesion and motility of cultured human KC, and specific pharmacological agents. Nicotinic agonists, including nicotine, increased motility and rate of cell attachment of cultured KC, while the specific antagonists κ -BTX and Mecamylamine caused cell detachment. The nicotinic cholinergic antagonists d-tubocurarine, hexamethonium, tetraethylammonium did not affect the motility and adhesion of cultured human KC.

In conclusion, human KC express AChRs similar or identical to ganglionic AChRs, which may include the $\alpha 3$, $\beta 2$ and $\beta 4$ subunits and which are involved in regulation of adhesion and cytoplasm motility. Since other non-excitabile cells synthesize and degrade ACh, and extrasynaptic AChRs are present in neurons, modulation of cell adhesion might represent a general primordial function of ACh, released in an autocrine or paracrine fashion, and mediated by nicotinic receptors.

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PRESYNAPTIC NICOTINIC RECEPTOR MODULATION OF DOPAMINE RELEASE IN RAT BRAIN

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Nicotine evokes dopamine release from rat corpus striatum *in vivo* and *in vitro* (Wonnacott *et al.* 1990). This effect is Ca^{2+} dependent and blocked by nicotinic antagonists (except at high nicotine concentrations) and is indicative of the presence of nicotinic acetylcholine receptors (nAChR) on dopamine terminals. Superfusion of striatal synaptosomes loaded with [³H]dopamine has been used to characterise the presynaptic nAChR. A detailed analysis of the dose-response relationship suggests that nicotine has a complex, biphasic effect consistent with receptor heterogeneity. Specific (mecamylamine-sensitive) nicotine-evoked [³H]dopamine release displayed two peaks of release, at 5 μM and 90 μM nicotine. Neither peak was sensitive to α bungarotoxin or methyllycaconitine (1 μM).

The presynaptic modulation of [³H]dopamine release provides a useful functional assay of brain nAChR. The physiological significance of these receptors is more controversial. Using *in vivo* microdialysis in conscious, freely moving rats, nicotine delivered into the striatum (ie the terminal areas) via the dialysis probe increases the concentration of endogenous dopamine recovered in a dose-dependent and mecamylamine sensitive manner. Thus presynaptic nAChR are capable of modulating dopamine release in the intact animal; evaluation of their influence relative to cell body stimulation requires comparison with the effects of nicotine administered into the substantia nigra.

To see if the presynaptic nicotinic stimulation of dopamine release is a general phenomenon, we have examined the effect of nicotine on [³H]dopamine release from frontal cortex synaptosomes. Nicotine elicited mecamylamine-sensitive release of tritium; the magnitude of the response was about one half of that seen in striatum. The dose-response profile conformed to a typical "inverted-U" shape, with a broad peak over the range 10-50 μM nicotine.

The *in vitro* effects of nicotine were compared in animals chronically treated with nicotine (4mg/kg/day for 14 days). This regime produced a significant (40%) increase in the density of [³H]nicotine binding sites in frontal cortex synaptosomes, compared with saline treated controls, whereas there was no significant increase in striatal preparations from nicotine treated animals. These data are in accordance with previous reports (Sanderson *et al.* 1993). Analysis of [³H]dopamine release evoked by 1 μM nicotine from the same preparations revealed a significant decrease in nicotine-evoked [³H]dopamine release in frontal cortex but no significant difference was observed in striatum.

	FRONTAL CORTEX		STRIATUM	
	³ H]nicotine binding sites (fmol/mg protein)	³ H]dopamine release (% control)	³ H]nicotine binding sites (fmol/mg protein)	³ H]dopamine release (% control)
Control	58±4	100	82±9	100
Nicotine-treated	80±7*	74±8**	103±8	92±8

* p<0.005
** p<0.05

For frontal cortex, these data indicate that sustained levels of nicotine upregulate numbers of [³H]nicotine binding sites but inactivate presynaptic nAChR function. Striatum appears less responsive to the effects of nicotine administration. This lower sensitivity to nicotine could reflect differences in the nAChR subpopulations or differences in the local mechanisms regulating them.

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CHOLINERGIC CHANNEL ACTIVATORS (ChCAs) FOR THE POTENTIAL TREATMENT OF ALZHEIMER'S DISEASE (AD)

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The existence of a diversity of putative neuronal nicotinic acetylcholine receptors (nAChRs) with a wide distribution in brain suggests that each subtype may be involved in mediating specific functions/behaviors, and each may have a defined pharmacology that can be selectively targeted (Arneric et al., 1995). Consistent with this concept, classical nAChR agonists (e.g. (-)-nicotine) bind to the acetylcholine binding site on the α subunit, while recent evidence suggests that neuronal nAChR function can also be enhanced via sites distinct from the agonist binding site. These sites are not subject to the same desensitization mechanisms described for (-)-nicotine (Arneric et al., 1995). Thus, a broader class of agents can be termed cholinergic channel activators (ChCAs), since they would include nAChR agonists as well allosteric modulators. The actions of these agents may occur via the selective interaction with central nAChR subtypes or some may act by positive allosteric modulation. Functionally, ChCAs selective for nAChR subtypes could enhance central neuronal nAChR mediated transmission without having the side-effect liabilities normally associated with (-)-nicotine. In particular, ChCAs lacking cardiovascular or other CNS side effects associated with (-)-nicotine may represent a potential therapeutic strategy to ameliorate many of the CNS deficits accompanying AD or other related disorders.

Increasing evidence suggests that compounds which activate neuronal nAChRs may have potential benefit in the treatment of dementia, especially Alzheimer's disease (AD) (for Review: Arneric and Williams, 1994). ABT-418 [(S)-3-methyl-5-(1-methyl-2-pyrrolidinyl) isoxazole], a novel analog of (-)-nicotine, is in clinical development for the treatment of AD. Based on preclinical studies, ABT-418 is a cholinergic channel activator (ChCA) with cognitive enhancement and anxiolytic-like activity possessing a substantially reduced side-effect profile compared to (-)-nicotine (Arneric et al., 1994; Decker et al., 1994). The enhanced preclinical safety profile and excellent transdermal delivery across species suggest that this compound may potentially be useful for the treatment of AD. Below is a synopsis of the preclinical pharmacology of ABT-418.

ABT-418 potently ($K_i = 3 \pm 0.4$ nM) inhibits high affinity [3 H](-)-cytisine binding, which is thought to represent an interaction with the $\alpha 4\beta 2$ subtype of nAChRs (Arneric et al., 1994), the major subtype in rodent brain. ABT-418 is significantly less potent ($K_i > 10,000$ nM) against the nicotinic receptors labeled by [125 I] α -bungarotoxin in rodent brain and neuromuscular junction. In 34 other receptor/uptake/ enzyme binding assays the K_i values for ABT-418 are greater than 10,000 nM (Arneric et al., 1994).

Functionally, ABT-418 (estimated $EC_{50} = 5$ μ M) is slightly less potent than (-)-nicotine (estimated $EC_{50} = 3$ μ M) to stimulate [3 H]ACh from rat hippocampal synaptosomes. Similarly, ABT-418 is less potent (estimated $EC_{50} = 6$ μ M), but 40% more efficacious than nicotine (estimated $EC_{50} = 1$ μ M) to evoke 86 Rb $^+$ efflux from mouse thalamic synaptosomes--an effect that is mecamylamine sensitive. This assay has been proposed to reflect the activation of nAChRs channel containing an $\alpha 4\beta 2$ configuration (Marks et al., 1993). In IMR 32 cells, cation efflux studies indicated that ABT-418 (estimated $EC_{50} = 100 \pm 20$ μ M) is a less potent activator of ganglionic-like nAChRs than (-)-nicotine (estimated $EC_{50} = 21 \pm 5$ μ M). In addition, ABT-418 activates nAChR channel currents in PC12 cells as evaluated by whole-cell patch clamp studies -- an effect prevented by the nAChR channel blocker mecamylamine (Arneric et al., 1994). PC12

cells also express ganglion-like nAChRs distinct from the $\alpha 4\beta 2$ subtype, and in this preparation ABT-418 is four fold less potent than (-)-nicotine (EC_{50} $214 \pm 30 \mu M$ vs. $52 \pm 4 \mu M$) in eliciting a functional response, which is consistent with the reduced cardiovascular liabilities seen in dog and monkeys. ABT-418 and (-)-nicotine stimulate the evoked release of [3H]dopamine from rat striatal slices with EC_{50} values of 380 ± 50 nM and 40 ± 10 nM, respectively (Arneric et al., 1994). The decreased potency of ABT-418 on dopamine release compared to (-)-nicotine agrees with *in vivo* drug discrimination studies in which ABT-418 does not fully generalize to (-)-nicotine (Brioni et al., 1994b). Thus, ABT-418 has functional selectivity for the major subtype of brain nAChR.

Fig. 1
Post-Training Administration of ABT-418
Enhances Performance of
Inhibitory Avoidance in Rats

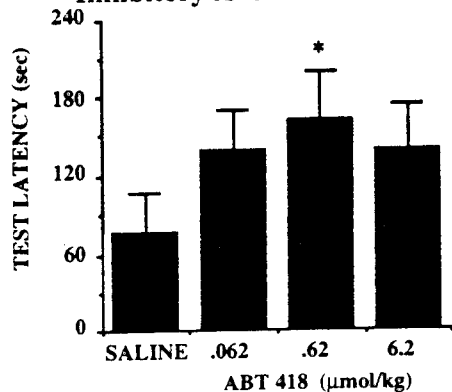
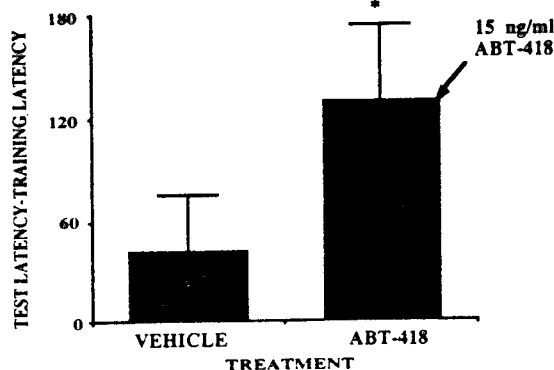
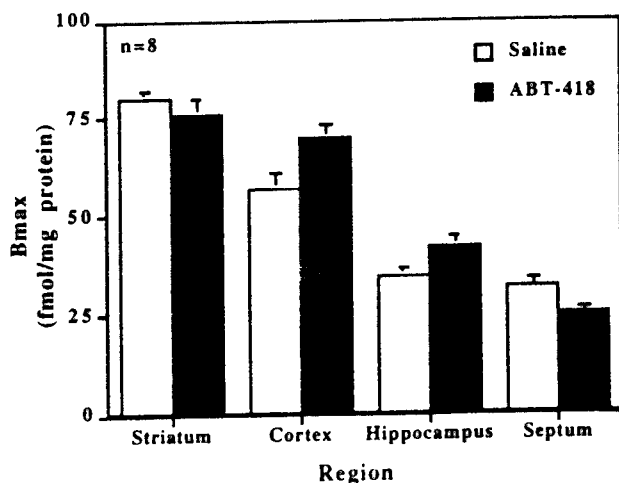


Fig. 2
Enhancement of Inhibitory Avoidance Performance
by ABT-418 is Sustained in Aged Rats
Continuous Infusion of ABT-418 with Alzet™ minipumps for 11 days



ABT-418 has been evaluated in a series of animal test paradigms to assess cognition enhancement. ABT-418 has a positive effect at $0.062 \mu\text{mol/kg}$ on the retention of inhibitory avoidance with pre-training injections in mice and at $0.62 \mu\text{mol/kg}$ with post-training injection in rats, *i.p.* (Fig. 1). These results suggest that ABT-418 may enhance memory consolidation. (-)-Nicotine produces similar effects at 3 to 10-fold higher doses (Decker et al., 1994a). The cognition enhancing activity of ABT-418 in this model in mice is prevented by mecamylamine ($5 \mu\text{mol/kg}$, *i.p.*). The effects are stereoselective since the (*R*)-enantiomer of ABT-418 was without effect.

Fig. 3
Levels of ABT-418 That Enhance
Cognitive Performance Do Not Alter
The Density of Neuronal nAChRs



Enhancement of performance is maintained in aged rats (20 months) following continuous infusion with osmotic minipumps over an 11-day treatment period (Fig. 2) without altering the total number of binding sites measured with [3H]-(-)-cytisine (Fig. 3). These results suggest that enhancement of cognitive performance is not attenuated ("desensitized") with chronic drug exposure, and that receptor numbers do not appear to be affected with low behaviorally effective circulating levels of ABT-418 (i.e. 15 ± 4 ng/ml). The *Morris water maze* paradigm is used to measure reference spatial memory in the medial septal lesioned rat. ABT-418, given *i.p.* (0.19 and $1.9 \mu\text{mol/kg}$) restores performance in a dose-related manner back to control levels (Decker et al., 1994b). The *delayed matching-to-sample task* assesses short term memory as well as attentive aspects of memory in primates. ABT-418 enhances

performance of this task in normal, young monkeys at an average maximally effective dose of 15 nmol/kg, i.m., whereas (-)-nicotine is effective at 19 nmol/kg, i.m. No adverse events are observed with ABT-418 at doses up to 500 nmol/kg following i.m. administration (Buccafusco et al., unpublished).

While ABT-418 has approximately the same potency as (-)-nicotine in memory tasks, the compound is remarkably less potent than (-)-nicotine in producing EEG activation, hypothermia, seizures, death, and reduction of locomotor activity in rodents. ABT-418 has significantly less emetic (Decker et al., 1994a) and pressor liability in dog as compared to (-)-nicotine (unpublished observation). In rodent, dog and monkey ABT-418 demonstrates substantial transdermal bioavailability, yet poor oral bioavailability due to rapid metabolism (A.D. Rodrigues, personal communication). Levels of ABT-418 within the range that elicited cognitive enhancement in the Morris water maze paradigm were maintained for up to 48 hours.

ABT-418 demonstrates anxiolytic-like activity in both mice and rats in the *elevated plus maze* model of anxiety at doses of 0.19 and 0.62 $\mu\text{mol/kg}$, i.p., respectively. ABT-418 is approximately 15-fold more potent than diazepam in mice, but is less efficacious in eliciting anxiolytic-like activity. Nonetheless, in contrast to diazepam, ABT-418 does not potentiate ethanol-induced narcosis, nor does it impair rotorod performance in the effective dose range (Brioni et al., 1994a; Decker et al., 1994a). Transdermal application of ABT-418 to rats via a Hill Top™ chamber elicits anxiolytic-like activity for at least 4 hours. The anxiolytic-like effect of ABT-418 in rats is blocked by mecamlamine (15 $\mu\text{mol/kg}$) (Brioni et al., 1994a).

ABT-418 is a prototype compound that selectively activates neuronal nAChRs without eliciting the dose-limiting side effects typically observed with (-)-nicotine. ABT-418 may be a safe and effective ChCA for the potential treatment of the cognitive and emotional impairments of AD.

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MODULATION OF CATALYSIS AND INHIBITION OF ACETYLCHOLINESTERASE BY MONOCLONAL ANTIBODIES

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Monoclonal antibodies (MAbs) that on complexation with an enzyme molecule alter its catalytic activity can serve as useful probes to define the mechanism of catalysis (Abe et al., 1983; Doctor et al., 1983; Brimijoin and Rakonczay, 1986; Ashani et al., 1990; Heider et al., 1991). Monoclonal antibodies have been raised against acetylcholinesterase (AChE) isolated from a variety of sources and species (Fambrough et al., 1982; Abe et al., 1983; Doctor et al., 1983; Brimijoin et al., 1985; Massoulié et al., 1988; Heider et al., 1991). Although it is obvious that none of these MAbs bind to the esteratic site (Ogert et al., 1990), some of them appear to interact with that region of the catalytic subunit referred to as the peripheral anionic site (Ashani et al., 1991).

In this report we describe the production and characterization of six inhibitory MAbs against fetal bovine serum (FBS) AChE. The results presented here show that changes in the conformation of AChE caused by interaction with MAbs at a site remote from the catalytic site result in the modulation of catalytic activity of AChE.

Four antibodies, 25B1, 4E5, 5E8, and 6H9, inhibited the catalytic activity of FBS AChE to >98% at ratios of 1-4 mole of MAb per mole of AChE. Two other anti-FBS AChE antibodies, 13D8 and 2A1, inhibited AChE approximately 70-80%. The percentage of inhibition did not increase with these two antibodies even though the ratio of antibody to enzyme was increased to 32:1. None of the six antibodies bound to or inhibited *Torpedo* AChE under the same conditions and with the same concentrations of enzyme and antibody. Only MAb 5E8 inhibited recombinant human AChE at AChE-MAb ratios of 1:16 (35% inhibition) and 1:32 (75% inhibition). They neither bound to nor inhibited horse serum BChE or human serum BChE. MAb 13D8, at a very high concentration (approximately 10 μ M), also recognized recombinant human AChE. Based on their inability to bind to reduced, denatured, and alkylated FBS AChE, it appears that all six MAbs have conformational epitopes.

Edrophonium and propidium reversibly inhibit AChE through competitive and noncompetitive associations with the enzyme, respectively. Previously, we showed that propidium, which binds to the putative peripheral anionic site (Taylor and Lappi, 1975), but not edrophonium, which binds to the catalytic site, retarded the rate of inhibition of FBS AChE by MAb 25B1 (Ashani et al., 1990).

The effect of edrophonium and propidium on the time course of the inhibition of FBS AChE by MAb 6H9 (Ashani et al., 1991) showed that the rate of AChE inhibition by this MAb is retarded by propidium. Although 25B1 and 6H9 form different types of complexes with FBS AChE and presumably bind to somewhat different regions on the surface of the catalytic subunit of the tetramer, the rate of inhibition of AChE by both of these MAbs is retarded by propidium.

Analysis of fractions from sucrose density gradient centrifugations of AChE:MAb complexes suggested that the MAbs could be categorized according to the apparent size of the complexes formed with the tetrameric form of FBS AChE. Based on these gradient data (and the stoichiometry of enzyme:antibody inhibition assays), two sizes of complexes could be distinguished: tetramers of enzyme cross linked by antibody molecules to form multimeric complexes (MAbs 13D8, 25B1, and 4E5) and discrete complexes of antibody molecules and single tetramers of enzyme that sedimented

as single peaks (Mabs 2A1, 5E8 and 6H9).

FBS AChE was titrated with four MABs using two different substrates. The complexes formed with increasing AChE:MAB ratio were assayed using ATC, a charged substrate, and indophenyl acetate (IPA), a neutral substrate. Complexes of MABs 25B1, 13D8, 2A1 and 4E5 with FBS AChE produced similar inhibition curves with both substrates. In contrast, with antibodies 6H9 and 5E8, the use of IPA caused a shift in the inhibition curve. In the case of 6H9, hydrolysis of IPA appeared to increase (1.25 fold) with increasing amounts of antibody, whereas hydrolysis of ATC was inhibited. A similar increase in IPA hydrolysis was shown to occur with MAB AE-2, raised against human erythrocyte AChE (Wolfe et al., 1993). Monoclonal antibody 5E8 inhibited ATC hydrolysis but had a marginal effect on IPA hydrolysis.

To further characterize the differences between various FBS AChE:MAB complexes, the extent of radiolabeling of the complex by phosphorylation with the neutral covalent inhibitor [³H]DFP was determined. In all cases, sufficient amounts of MAB were used to attain maximum inhibition of catalytic activity. The extent of radiolabeling of complexes was determined following 24 hrs of incubation at 25°C with a 2.25-fold molar excess of [³H]DFP. Results indicated that the nucleophilicity of the active-site serine of the various MAB:AChE complexes had been altered.

Measurement of displacement of the organophosphate (OP) moiety from all [³H]DFP:AChE:MAB complexes by the oxime TMB₄ showed that two of the MAB-bound DFP:AChE complexes, those with antibodies 25B1 and 13D8, lost nearly all radioactivity when incubated with 1 mM TMB₄. By contrast, the other four MAB:DFP:AChE conjugates lost smaller amounts of radioactivity, 25-50%, when incubated with 1 mM TMB₄. TMB₄, however, was able to reach the catalytic site of the enzyme in all complexes and to dephosphorylate the enzyme but to different levels.

Although attempts have been made to generate MABs that alter the enzymatic activity of AChEs (Brimijoin and Rakonczay, 1986), to date only a few MABs are available which inhibit the catalytic activity (Abe et al., 1983; Brimijoin et al., 1985; Sorensen et al., 1987; Doctor et al., 1989; Ashani et al., 1990). When fusions using various forms and modifications of AChE (i.e., reduced, denatured, and alkylated AChE) and organophosphate-inhibited AChE as immunogens were attempted, only native forms of AChE and OP-inhibited AChE resulted in the production of inhibitory MABs. The fact that all inhibitory antibodies obtained to date were generated by the native or OP-inhibited form is consistent with the fact that their epitopes are conformational. Since the antibodies raised against phosphorylated enzyme have, in general, similar inhibitory characteristics to the antibody against the native form, this also provides anecdotal evidence that phosphorylation of the cholinesterase does not appear to produce a major change in the enzyme's surface. That all antibodies are of the IgG class also extends their usefulness, since in our experience, IgM antibodies, because of their size, are more difficult to purify and to use.

The results also indicate that the nucleophilicity of the O^γ atom of the catalytic serine in AChE:MABs varies. The fact that oxime TMB₄ was able to reach the catalytic site and cause displacement of the organophosphoryl-bound moiety from the complexes but to different levels implies that the entrance to the active site gorge either has not been blocked (13D8 and 25B1) or is somewhat restricted. This can be attributed to conformational changes, steric hindrance, or a combination of both factors.

Trp279 (*Torpedo* AChE numbering system), which resides at the entrance to the gorge of all AChEs, has been implicated as part of the binding region of peripheral ligands (Shafferman et al., 1992; Radić et al., 1993). The fact that the peripheral anionic site ligand propidium retarded the binding of all MABs to FBS AChE suggested that this amino acid may be a common determinant of

their epitopes. However, since these MAbs do not cross react with *Torpedo* and human AChEs (except in the case of 13D8 at high concentrations), it appears that there are other critical residues that constitute their binding sites.

Wolfe et al. (1993) have reported that MAb AE-2, directed against human RBC AChE and cross reacting with FBS AChE (Fig. 1), inhibits the catalytic activity of AChE, when the charged substrate ATC is used, but stimulates the hydrolysis of neutral substrate IPA, as does MAb 6H9. They suggested that AE-2 may allosterically modulate an anionic subsite in the catalytic center of FBS AChE (Trp84). Additionally, Wasserman et al., (1993), Ogert et al. (1990), and Doctor et al. (1989) have previously shown that epitopes for several MAbs generated against AChEs are located on the surface of the catalytic subunit in the region of amino acids 40-100. The role of Trp84 as an anionic subsite has been established (Harel et al., 1992; Shafferman et al., 1992; Radić et al., 1993), and it has been suggested that it and Met83 are partially exposed on the surface of the molecule (Harel et al., 1992; Gilson et al., 1994). This region appears to be highly immunogenic. The fact that MAb 6H9 and, to a lesser extent 5E8, when complexed with AChE stimulated the hydrolysis of IPA, as does AE-2, suggests that this highly immunogenic region may also be a binding site for these two MAbs. The possibility exists that these MAb combining sites can span both of these regions (B.P. Doctor and J. L. Sussman, personal communications).

The results suggest that different MAbs modulate the activity of FBS AChE via binding to a site remote from the catalytic region of the enzyme. However, the relative contribution of the MAbs to the issue of conformational changes vs. steric hindrance awaits further study.

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*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

THURSDAY, NOVEMBER 9, 1994

(MORNING, SECOND SESSION)

CHOLINESTERASE I

Chair: Dr. B. Sakmann; Co-Chair: Dr. R. Balleit

10:50	Silman, I.	Studies of Partially Unfolded States of <i>Torpedo californica</i> Acetylcholinesterase
11:10	Shafferman, A.	Molecular Aspects of Catalysis and of Allosteric Regulation of Acetylcholinesterase
11:30	Taylor, P.	Expression and Ligand Specificity of Acetylcholinesterase and the Nicotinic Receptor: A Tale of Two Cholinergic Sites
12:00	Massoulié, J.	Molecular Forms of Acetylcholinesterase: Structure and Interactions
12:30	Sussman, J.	3-D Structure of Acetylcholinesterase and Complexes of it with Anticholinesterase Agents
13:00	--	Lunch Break

STUDIES ON PARTIALLY UNFOLDED STATES OF *TORPEDO CALIFORNICA* ACETYLCHOLINESTERASE

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Chemical modification, by a repertoire of thiol reagents, of the non-conserved Cys²³¹ residue of *Torpedo californica* AChE, results in inactivation, even though Cys²³¹ is not involved in catalysis (Steinberg *et al.*, 1990; Dolginova *et al.*, 1992; Silman *et al.*, 1992; Salih *et al.*, 1993). Modification by disulfides and alkylating agents produces partial unfolding of native AChE (N) to a compact state resembling a molten globule (MG). The MG is a collapsed structure possessing much of the secondary structure of the fully folded native protein, but devoid of tertiary structure (Kuwajima, 1989; Ptitsyn, 1992); it is currently believed to serve as a folding intermediate *en route* from the nascent polypeptide chain, generated on the ribosome, to the fully folded native protein (Kim and Baldwin, 1990; Gething & Sambrook, 1992). This structural assignment for the species produced by chemical modification of *Torpedo* AChE is based upon spectroscopic evidence, including CD, intrinsic fluorescence and binding of ANS, upon hydrodynamic measurements, including sucrose gradient centrifugation and quasielastic light scattering, and upon enhanced sensitivity to proteolysis. Although modification by disulfides can be rapidly reversed by exposure to reduced glutathione (GSH), the native (N) conformation is not restored, and no catalytic activity is recovered. AChE so demodified is a partially unfolded species whose physicochemical characteristics are virtually identical to those of the modified enzyme, and sucrose gradient centrifugation reveals it to be stable for many hours without aggregating.

Chemical modification by mercurials also inactivates *Torpedo* AChE. However, such modification produces a N-like state, *viz.* a conformational state much closer to N, although also devoid of catalytic activity. Upon demodification with GSH of *Torpedo* AChE freshly modified with mercurials, up to 85% of the initial AChE activity is recovered, with full restoration of the spectroscopic characteristics of N. However, the N-like state produced by mercurials is not stable; it unfolds spontaneously, with a $t_{1/2}$ of *ca.* 1 hour, to a non-reactivable, partially unfolded form which is similar to that produced by the disulfides and alkylating agents. Arrhenius plots show that the N-like state is separated by a low (5 kcal/mol) energy barrier from the N state, whereas the partially unfolded, MG-like state is separated from the N-like state by a high-energy barrier (*ca.* 50 kcal/mol).

Comparison of the 3-D structures of native *Torpedo* AChE and of a heavy-atom derivative obtained with HgAc₂ (Sussman *et al.*, 1991), suggested a basis for the stabilization produced by the mercurials relative to other thiol reagents: the mercurial-modified enzyme may be stabilized by additional interactions of the mercury atom attached to Cys²³¹, specifically with Ser²²⁸Oγ and with the main-chain nitrogen and carbonyl oxygen of the same serine residue.

The reason why AChE remains in the partially unfolded state produced by chemical modification, even after modification has been reversed, is not yet understood. Two explanations may be offered for this phenomenon. The first explanation would involve kinetic trapping. It would assume that, even though the N state might be at a lower free energy-level than the MG-like state, they are separated by a high free-energy barrier. Examples of such kinetic trapping have appeared in the literature, one of the best known being that of α-lytic protease (Baker *et al.*, 1992), for which a partially unfolded state will not fold back to the native conformation in the absence of a pro-region. AChE, however, does not contain a pro-region (Maulet *et al.*, 1990). A high energy barrier, *ca.* 20 kcal/mol, is observed in protein transitions involving isomerization of proline (Koide *et al.*, 1993), and there are two proline residues, Pro²²⁸ and Pro²³², adjacent to Cys²³¹, both of which are in the *trans* conformation (Sussman *et al.*, 1991). It is possible that the chemical modification of Cys²³¹ 'drives' one or both of these prolines to a *cis* conformation, thus providing a high energy barrier to the reverse transition. Since *Torpedo* AChE is a large protein (a dimer of two identical subunits, with 537 residues each), it is also possible that non-native domain pairing and/or intersubunit interactions are responsible for trapping AChE in the partially unfolded state (Jaenicke, 1991).

A second explanation would be that the N state of *Torpedo* AChE does not correspond to the global free-energy minimum, and that the energy of the MG-like state is, in fact, lower than that of N. This seems plausible, since the quasi-N state produced by mercurials cannot be energetically very different from the native enzyme, yet it converts spontaneously to its partially unfolded MG-like counterpart. This, in turn, cannot be at an energy level very different from that of the demodified enzyme (or the analogous demodified enzyme obtained after modification with disulfides), since their spectroscopic properties are quite similar. A recent theoretical paper has indeed made the point that 'identification of native states with the most compact or minimum energy states may not strictly hold' (Bahar and Jernigan, 1994).

Since the stable states produced by chemical modification display many of the features of the MG, we also produced such states of AChE by 'traditional' methods, e.g. by exposure to 1.2 M guanidinium chloride (Gu). In 1.2-2.1 M Gu, AChE is in a MG state, but the N→MG transition is irreversible; upon removing Gu, no enzymic activity is detected, and the spectroscopic characteristics of N are not recovered. In 5 M Gu, AChE is

in an unfolded state (U), in reversible equilibrium with MG. If Cys²³¹ is labelled selectively, with a mercury derivative of a stable nitroxyl radical, it can be seen that the EPR signal of the MG state is highly immobilized, whereas in the U state the probe is almost freely rotating. It is thus possible to show that they co-exist in the transition region and to measure their relative amounts. Upon elevating the Gu concentration, a decrease in the EPR signal corresponding to the MG state occurred concomitantly with an increase in that of the U state, the integral intensity of the EPR spectra remaining constant. Such behavior is characteristic of a two-state transition. The thermodynamic characteristics of the transition, whether estimated directly from the EPR data, or from both CD and fluorescence measurements, assuming a two-state scheme, are in good agreement. There has been considerable controversy, at both the theoretical (Alonso *et al.*, 1991; Finkelstein & Shakhnovich, 1991) and experimental (*cf.* Shimizu *et al.*, 1993; Uversky, 1993) levels, as to whether the MG \leftrightarrow U transition can, indeed, be described by a two-state model. Our data clearly demonstrate this to be the case for AChE, and show that EPR can serve as a powerful tool in monitoring such transitions.

Exposure of purified *Torpedo* AChE to a system generating oxygen radicals (*viz.* ascorbic acid/Fe(EDTA)₂H₂O₂) led to inactivation. The enzyme retained its dimeric form, but electrophoresis under denaturing conditions revealed some cleavage of peptide bonds. Spectroscopic examination showed that the partially inactivated enzyme displayed spectral properties resembling those of the MG-like state produced by chemical modification, and that it also displayed enhanced susceptibility to proteolysis (Weiner & Silman, 1993; Weiner *et al.*, 1994). These observations may provide a model system for understanding the consequences of oxidative stress *in vivo*. We propose that partially unfolded proteins, generated by oxidative stress, may interact with molecular chaperones of the heat-shock family, thus leading to release of the heat-shock transcription factor, and to activation of heat-shock genes, as has been shown to occur in the heat-shock response (Morimoto, 1993; Matts *et al.*, 1993). The heat-shock proteins so generated could then combine with the misfolded proteins and protein fragments produced by oxidative stress, and eliminate them from the cell by transport into lysosomes, followed by degradation (Chang *et al.*, 1989). Our data thus suggest a molecular basis for the overlapping regulation of heat shock and oxidative stress which has been noted by a number of laboratories (*cf.* Tartaglia *et al.*, 1991; Keyse & Emslie, 1992).

It has been proposed that the MG may serve not only as a folding intermediate in the biosynthesis of proteins, but also in their translocation across or insertion into plasma membranes (*cf.* van der Goot *et al.*, 1991). As mentioned above, the MG-like states of *Torpedo* AChE which we have generated are stable for many hours, under physiological conditions, without undergoing aggregation. They thus provide an experimental system for investigating MG interactions with lipid bilayers. Indeed, in preliminary experiments, using a flotation gradient technique, we have been able to demonstrate rapid insertion of

AChE in the MG-like state into dimyristoylphosphatidylcholine liposomes, whereas native AChE displays no such interaction. 1 M NaCl does not significantly decrease the interaction, showing that electrostatic forces do not play a major role. It was also observed that interaction of the MG with the liposomes causes slow leakage of fluorescent (calcein) or spin (TEMPO-choline) probes which had been pre-loaded into the liposomes.

Our ability to generate partially unfolded, stable states of *Torpedo* AChE, by either chemical or physical manipulation, thus provides a model system which permits an experimental approach to several problems of current interest to protein chemists, to membrane biophysicists and to cell biologists.

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Molecular Aspects of Catalysis and of Allosteric Regulation of Acetylcholinesterases.

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Introduction

Acetylcholinesterase is a serine hydrolase whose function at the cholinergic synapse, is the rapid hydrolysis of the neurotransmitter acetylcholine (ACh). The recently resolved 3D structure of *Torpedo californica* AChE (TcAChE) revealed a deep and narrow 'gorge', which penetrates halfway into the enzyme and contains the catalytic site at about 4Å from its base (Sussman *et al.*, 1991). The active center interacts with ACh through several subsites including the catalytic triad (Ser203(200), His447(440), Glu334(327); Sussman *et al.*, 1991; Gibney *et al.*, 1990; Shafferman *et al.*, 1992a,b), the oxyanion hole (Gly121(119), Gly122(120), Ala204(201); Sussman *et al.*, 1991), the acyl pocket (Phe295 (288) and Phe297(290); Vellom *et al.*, 1993; Ordentlich *et al.*, 1993a).

The nature of the fourth element of the active center, the anionic subsite, is a matter of a longstanding controversy. One opinion, argued on the basis of the alleged presence of multiple negative charges in the active center, that the "anionic subsite" is a true anionic locus (Quinn, 1987). The opposite view based on the structure - activity studies with charged and noncharged substrates and inhibitors suggested that the "anionic subsite" is in fact a trimethyl site, binding the ligands through hydrophobic interactions (Hassan *et al.*, 1980) or dispersive forces (Nair *et al.*, 1994). We will provide evidence that the "anionic subsite" is not a true ionic site and that quaternary ammonium of ACh is stabilized by cation-aromatic interaction with Trp-86 at the active center.

AChE is an extremely efficient enzyme, believed to operate at, or near the diffusion control limit (Quinn, 1987). Recently, it was shown that in the structure of TcAChE an uneven spatial charge distribution results in a negative electrostatic potential that may be involved in the attraction of the positively charged substrate into the active center gorge of the enzyme (Tan *et al.*, 1993; Ripoll *et al.*, 1993). However the actually measured bimolecular rate constants of various AChEs are about an order of magnitude lower than the expected rates of diffusion controlled reactions and furthermore they appear to be dependent on the molal volume of the various substrates rather than on charge (Cohen *et al.*, 1984). To further investigate the proposed contribution of electrostatic attraction to the rate of catalytic activity of AChE, we neutralized by mutagenesis up to seven of the surface negative charges, located near the rim of the active site gorge of AChE. We will show that while such substitutions have dramatic effects on the electrostatic potential, no major effects on the reactivity towards various substrates were observed.

A remarkable feature of AChE is the capacity to bind structurally diverse cationic ligands. Some of these ligands bind at the active center while others associate with a peripheral anionic site (PAS), remote from the active center (for reviews see Hucho *et al.*, 1991; Massoulié *et al.*, 1993, Taylor and Radic 1994). Allosteric modulation of AChE catalytic activity, through binding to the PAS, was first suggested by Changeux (1966). The resolution of the enzyme structure, by x - ray crystallography (Sussman *et al.*, 1991), did not yield any clues as to the mechanism of the allosteric effect but rather rendered it more enigmatic by placing the active site within a narrow gorge about 20Å away from the periphery. By employing site directed mutagenesis, molecular modeling techniques and fluorescence binding studies we generate information pertaining to the topography of the complexes of HuAChE with classical PAS ligands. We demonstrate that alternative residues participate in interaction with various ligands and provide some insight into the allosteric modulation of AChE activity.

Results and Discussion

The quaternary ammonium moiety of AChE ligands is stabilized in the active center by Trp-86 through cation- π interactions.

Examination of the x-ray structure of TcAChE (Sussman *et al.*, 1991) and of the derived model of HuAChE (Barak *et al.*, 1992), reveals that the only negatively charged residue vicinal to the catalytic serine is Glu-202. The two other acidic residues: Asp-74 and Glu-450, located within the active site gorge, are 15.1Å and 8.9Å away from residue Ser-203 respectively and are therefore unlikely to participate in interactions of the "anionic subsite". Substitutions of Glu-202 of the human AChE (HuAChE) enzyme by neutral residues (Ala or Gln) or by Asp exerts a comparable effect on catalysis for both acetylthiocholine (ATC) and its noncharged isostere TB. These results suggest that residue Glu-202 has no specific role in stabilizing positively charged substrates and therefore is not a part of the "anionic subsite". On the other hand, the kinetic data clearly indicate that the correct positioning of the negative charge of Glu-202 plays an important role in the acylation step of the catalytic reaction as well as in phosphorylation and "aging" (Shafferman 1992b, Vellom *et al.* 1993, Ordentlich *et al.* 1993b).

Unlike the indiscriminative effect on catalysis towards charged and noncharged substrate due to replacement of Glu-202, substitutions at position 86 affect differentially the hydrolytic activity for the two kinds of substrates. The bimolecular rate constant of the wild type enzyme for ATC is 20-fold higher than that for TB, yet in the HuAChE mutants carrying aliphatic residues at position 86 the selectivity is reversed and these enzymes show 50-fold higher reactivity for TB than that for ATC. This reversal of selectivity towards the sterically identical noncharged substrate, and the fact that kinetic parameters for TB are only marginally affected by the various mutations, is a clear manifestation of the existence of a functional "anionic subsite" and of the role of residue Trp-86 in this subsite. Substitution of Trp-86 by the aliphatic residues alanine or glutamate, but not by phenylalanine, brings about 600-fold increase in the Michaelis Menten constant for the charged substrate ATC and an overall decrease of 3000-7000-fold in the apparent bimolecular rate constant. These results underscore the importance of aromatic residue at position 86 in stabilizing the Michaelis Menten complexes of HuAChE with charged substrates. Such conclusion is also supported by : a. The lack of measurable affinity of W86A and W86E mutants towards the charged active center inhibitor edrophonium; b. The 8500 and 15000-fold increase, relative to the wild type enzyme, in inhibition constant (K_i) value for decamethonium in the W86A and W86E enzymes respectively; c. The hundred fold higher affinity of W86F HuAChE towards edrophonium or decamethonium, compared to either the W86A or the W86E enzymes. In marked contrast, the nature of residue at position 86 has only a marginal contribution to the activity of HuAChE towards noncharged substrates like TB or noncharged inhibitors like DFP. Taken together these observations imply also that in the Michaelis Menten complexes, the orientations of the trimethyl ammonium and the 3,3-dimethylbutyl groups, of ATC and TB respectively, are not equivalent relative to Trp-86.

In conclusion, results from site directed mutagenesis studies of HuAChE and from x-ray crystallography of TcAChE (Sussman *et al.*, 1991) and its complexes (Harel *et al.*, 1993) provide a compelling evidence for the presence of a specific site which stabilizes the quaternary ammonium groups of substrates and other ligands through cation-aromatic interactions, mainly with residue Trp-86, rather than through ionic interactions.

Electrostatic attraction by surface charge does not contribute to the catalytic efficiency of acetylcholinesterase.

The structures of acetylcholinesterases are characterized by a high net negative charge (e.g. -11e for HuAChE; -12e for bovine AChE or -14e for TcAChE) and by asymmetrical distribution of acidic and basic amino acids on the protein surface, with excess acidic residues in the "northern" hemisphere. Conservation of an excess negative charge, in the vicinity of the

entrance to the active site gorge, taken together with the fact that the natural substrates bear a positive charge, may suggest that the electrostatic properties are a part of an evolutionary design for optimization of the catalytic efficiency of cholinesterases. Indeed, recent evaluation of the possible effects of the shape of TcAChE and its charge distribution, on the diffusion controlled rate of enzyme-substrate encounter by numerical brownian dynamics simulation, suggested an over 80 - fold rate enhancement (Tan *et al.*, 1993). According to this simulation, most of the enhancement (over 40 - fold) is due to electrostatic attraction, while a further minor effect could be attributed to electrostatic steering effects. Consequently, the uneven surface charge distribution and the resulting electrostatic potential, extending over the 'northern' hemisphere of AChEs, was proposed to contribute to the high catalytic efficiency of these enzymes (Ripoll *et al.*, 1993; Tan *et al.*, 1993).

This hypothesis was initially tested through simulated modulation of the charge distribution, in the "northern" hemisphere of HuAChE, and examination of the effects on the electrostatic potential as an indicator of the enzyme capacity for electrostatic attraction. Four out of the eleven acidic amino acids that constitute the net negative charge of the enzyme, are located within the active site gorge and do not contribute significantly to the electrostatic potential above the surface. Neutralization of remaining 7, or even 6 negative surface charges, practically abolishes the negative electrostatic potential over most of the "northern" hemisphere. Moreover, the direction of the electric field, which in the wild type HuAChE is aligned along the active site gorge axis, as observed also for TcAChE (Ripoll *et al.*, 1993), changes by $\sim 20^\circ$ away from the z-axis. It was therefore expected that an actual neutralization of the surface negative charges should affect the bimolecular rate constant of the enzyme - substrate reaction, provided that it does indeed depend on electrostatic attraction. This was examined, through generation of 20 HuAChE enzymes, mutated in up to seven acidic amino acids, vicinal to the rim of the active site gorge. In marked contrast to the shrinking of the electrostatic potential, the kinetic constants for reactivity of the mutants towards charged substrate and inhibitor are practically invariant, indicating that the electrostatic attraction does not contribute to the reaction rates.

The values of the apparent first order rate constant of catalysis (k_{cat}) of both ATC and TB are invariant for all the HuAChE mutants and are not sensitive to the ionic strength of the medium. This finding indicates that the active sites of the various mutants are effectively shielded from surface charges. Furthermore, it suggests that long-range electrostatic interactions, due to the surface charges of AChE, do not participate in stabilization of transition states in the catalytic process. The lack of contribution of electrostatic attraction to the catalytic rate, together with the nature of its dependence on ionic strength suggest that the rate of enzyme - substrate reaction is not diffusion controlled.

Our results, showing unequivocally the lack of contribution of electrostatic attraction to AChE catalytic properties (Shafferman *et al.* 1994), underscore the enigma of the uneven charge distribution, conserved throughout the cholinesterase family. It is possible that the electrostatic attraction in aqueous solutions is cryptic while in the viscous milieu of the synaptic cleft it becomes operational. Alternatively, the uneven charge distribution may be related to the noncatalytic functions of cholinesterases such as non - synaptic neuronal function, development of the nervous system or cell adhesion.

Identification of peripheral anionic site (PAS) elements and their involvement in allosteric modulation of AChE activity.

The functional significance of PAS is a controversial issue. This site was implicated in the catalytic pathway of acetylcholine (ACh) hydrolysis, in ionic strength monitoring and in substrate inhibition. A decrease in affinity for a selective PAS ligand (propidium) and a concomitant loss of substrate inhibition, were observed for certain human AChE mutants (Shafferman *et al.*, 1992b). Determination of the structural parameters affecting ligand-affinity for the PAS can have important implications for the design of anti - AChE drugs. A number of such drugs are under clinical trials while additional AChE inhibitors are investigated as a possible treatment for Alzheimer's disease. Site directed labelling (Weise *et al.*, 1990) and

mutagenesis studies (Shafferman *et al.*, 1992a,b; Ordentlich *et al.*, 1993) allow for an approximate localization of PAS at or near the rim of the active center 'gorge'. Such topography is compatible with the suggestion that bisquaternary ligands, typified by decamethonium, bind to the enzyme by bridging the active and the peripheral sites (Krupka, 1966).

Several of the residues constituting the PAS in HuAChE were recently identified by kinetic studies of 19 single and multiple HuAChE mutants, fluorescence binding studies and by molecular modeling (Barak *et al.* 1994). Mutated enzymes were analyzed with three structurally distinct positively charged PAS ligands- propidium, decamethonium and di(p-allyl-N-dimethylaminophenyl) pentane-3-one (BW284C51), as well as with the selective active center inhibitors - hexamethonium and edrophonium. Single mutations of residues Tyr-72, Tyr-124, Glu-285, Trp-286 and Tyr-341, resulted in up to 10-fold increase in inhibition constants for PAS ligands, whereas for multiple mutants up to 400-fold increase was observed. The sixth PAS element residue Asp-74, is unique in its ability to affect interactions at both the active site and the PAS (Shafferman, *et al.*, 1992b) as demonstrated by the several hundred-fold increase in K_i for D74N inhibition by the bisquaternary ligands decamethonium and BW284C51. Cooperativity of multiple mutations of PAS residues including Asp-74 may lead to over 10,000 fold increase in inhibition constants. Based on these studies, singular molecular models for the various HuAChE-inhibitor complexes were defined. Yet, for the decamethonium complex two distinct conformations were generated, accommodating the quaternary ammonium group by interactions with either Trp-286 or with Tyr-341. We propose that the PAS consists of a number of binding sites, close to the entrance of the active site gorge, sharing residues Asp-74 and Trp-286 as a common core. This functional degeneracy is a result of the ability of the Trp-286 indole moiety to interact either via stacking, aromatic-aromatic or via π -cation attractions and the involvement of the carboxylate of Asp-74 in charge-charge or H-bond interactions.

The influence of Asp-74 substitution, on inhibition by both peripheral site and active center specific ligands and in particular its striking effect on ligands bridging the two sites, is consistent with its proposed role (Shafferman *et al.*, 1992b) in the relay of allosteric signals from the periphery. Interestingly, replacement of residues Tyr-72, Tyr-124, Trp-286, Glu-285 and Tyr-341 generates AChE molecules in which substrate inhibition is affected to about the same extent as the inhibition constants for PAS ligands (up to 10-fold). Yet, unlike for inhibition by PAS ligands, no synergistic effects on substrate inhibition were observed, for the multiple mutants studied. Similar results were also reported by Radic *et al.* (1994). This may indicate that the substrate interacts at multiple locations within the PAS array. Existence of two binding loci within this array, were suggested above for the quaternary ammonium group of decamethonium, which structurally resembles the cationic head of the substrate.

Compared to the wild type enzyme the hydrolysis of ATC by the W86A mutated enzyme is almost completely refractive to inhibition by the PAS inhibitor propidium. These observations are quite surprising in view of the location of Trp-86 deep inside in the active site gorge, 15Å away from the surface of the enzyme and the binding site of propidium. To account for this and other results we proposed a functional cross-talk between Trp-86 and the peripheral anionic site (Shafferman *et al.* 1992b, Ordentlich *et al.* 1993a). This conclusion is consistent with spectroscopic studies by Berman *et al.*, (1981), and Berman and Nowak (1992) that demonstrated that occupation of the peripheral anionic site affects the conformation of the active center. More recent molecular models of the wild type, and of some mutated HuAChE enzymes reveal that the side chain of residue Trp-86 can occupy two conformational states. It is proposed that allosteric modulation of AChE activity, induced by binding to the peripheral anionic sites, proceeds through conformational transition of Trp-86 from the state of a functional "anionic subsite" to one that restricts access of substrates to the active center. Although the conformational flexibility of Trp-86 and its effects on the catalytic activity provide a possible mechanism for the "cross - talk" between the peripheral sites and the active center, the relay path of the allosteric signal still remains a matter for speculations.

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EXPRESSION AND LIGAND SPECIFICITY OF ACETYLCHOLINESTERASE AND THE NICOTINIC RECEPTOR: A TALE OF TWO CHOLINERGIC SITES

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Acetylcholinesterase (AChE) and the nicotinic acetylcholine receptor (nAChR) are the major functional proteins controlling postsynaptic events at the neuromuscular junction and in certain other central and peripheral nervous system synapses. In addition to AChE's and nAChR's common recognition capacity for acetylcholine and certain inhibitors, both proteins show proximal localization in synapses, function in a millisecond time frame and exhibit common features of expression during differentiation in muscle or following denervation [1,2]. The fidelity of neuromuscular transmission in the organism and the duration of miniature end-plate potentials in individual synapses require that the active states of these proteins be functioning within close stoichiometric confines. In autoimmune myasthenia gravis, functional receptor is diminished and the disease can be managed by prolonging acetylcholine in the synapse with AChE inhibitors. AChE inhibition by insecticides and a congenital myasthenic state with diminished endplate AChE also lead to compromised synaptic neurotransmission.

Comparative sequences [3] and an X-ray crystal structure [4] enable one to examine structure of AChE and its inhibitor complexes at an atomic level of resolution, whereas the nAChR's disposition within integral membranes makes crystallization a formidable endeavor. Nevertheless, electron microscopy image analysis [5] has provided structural details of the nAChR approaching 10-15Å resolution.

For AChE and the nAChR, some general structural features prevail for stabilization of the quaternary ammonium moiety of bound ligands. However, the noteworthy comparative aspects for these proteins are not a result of evolutionary divergence of related structures or convergence to a common motif, but rather emerge from parallel, independent evolutionary pathways that ultimately coordinate specificity and gene expression.

Gene Expression of the Nicotinic Receptor and Acetylcholinesterase

The nicotinic receptor family of receptors exists as ligand-gated ion channels assembled as pentamers of homologous subunits. Since as many as four different subunits are found in the pentamers and at least 16 different nicotinic receptor subunits have been identified, the possibility for receptor diversity is enormous. Since each gene encoding these subunits has a distinct promoter region, transcription might be expected to be a dominant, differential regulatory mechanism of gene expression.

By contrast, AChE is encoded by a single gene and its structural variations arise from alternative mRNA splicing yielding three distinct carboxyl-termini. The portion of the gene encoding the essential catalytic residues is invariant, giving rise to identical catalytic properties for the molecular species. The distinct carboxyl-termini allow the enzyme to be expressed as: (a) hydrophilic monomers, (b) homomeric dimers and tetramers which vary in hydrophobicity through attachment of a fatty acid or exposure of an amphipathic helix, (c) a glycopospholipid-linked species, and (d) heteromeric oligomers which disulfide link to a filamentous, triple-helical collagen unit or a lipid-linked subunit. RNA splicing governs the structural diversity of AChE, but multiplicity of species is further enhanced by post-translational events [6,7].

A second difference between AChE and the nicotinic receptor lies in the control of gene expression. Upon differentiation from myoblasts to myotubules,

the enhanced expression of the nAChR gene is a consequence of increased transcription [8]. By contrast, we find enhanced AChE gene expression in the same differentiation scheme is due to stabilization of a rapidly turning over mRNA [9]. Decreased degradation of AChE mRNA with a concomitant increase in mRNA levels appears to be controlled, at least in part, by the release of cellular Ca^{2+} (Luo, Z. et al., submitted). Ca^{2+} has little influence on the increased expression of the nAChR during muscle differentiation. Only AChE expression shows superinduction after initiation of differentiation of the myoblast and subsequent treatment with cycloheximide [9].

The distinction of transcriptional activation versus mRNA stabilization is evidenced by comparing: (a) transcription rates in myoblasts and myotubes by run-on transcription, (b) transcription rates of reporter genes placed behind the nAChR and AChE promoters, and (c) differential rates of gene expression in cell lines containing or lacking the muscle determination genes.

Ligand Specificity at Cholinergic Binding Sites

The most notable difference between AChE and the nAChR, as two oligomeric proteins, is that the agonist sites in the receptor reside between subunit interfaces, whereas AChE's active center is close to the geographic center of each subunit at the base of a 18-20Å gorge. A site at the interface between subunits for ligand binding to the nAChR accords with the cooperativity seen for ligand-gated channel opening and for ligand binding. By contrast, each AChE subunit appears to behave in an independent, non-cooperative fashion.

The crystal structure, sequence comparisons and mutagenesis studies in AChE allow one to define three distinct domains responsible for ligand specificity. Two lie within the active center. The first constitutes the acyl pocket which is formed by two phenylalanine [F_{295} and F_{297}] side chains pointing towards the gorge. They permit substrates with small acyl groups (i.e. acetylcholine and small alkylphosphates) to interact with AChE but not substrates containing large acyl groups (i.e. butyrylcholine, benzoylcholine and isoOMPA). F_{295} and F_{297} in mouse AChE exist as Leu (L) and Ile (I), respectively, in butyrylcholinesterase (BuChE). The $F_{295}L$ substitution is the primary means for achieving BuChE substrate specificity in an AChE template. Accordingly, we can expect the butyryl moiety to be directed to the 295 side chain. AChE and BuChE also differ in showing substrate inhibition and activation, respectively, in the presence of excess substrate. The 297 position appears to be the primary determinant of substrate inhibition and activation behavior. $F_{297}I$ substitution in an AChE template is sufficient to convert the substrate inhibition seen in AChE to substrate activation.

The other active center locus is in the choline binding subsite which is formed by W_{86} , Y_{341} , Y_{449} , F or Y_{337} and E_{202} . Except for the substitution $Y_{337}A$, these residues are identical in BuChE. The crystal structure of edrophonium and acridine complexes of AChE [10] and energy minimization with docking of acetylcholine show the aromatic residues to surround the choline and contribute to the stabilization energy. However, an anionic side chain from E_{202} comes within 1-2Å of the van der Waals radius of bound quaternary (trimethylammonio) groups. Both the Coulombic and hydrophobic interactions (dispersion forces) have been touted as primary contributors to the stabilization energy, but an analysis of free energy differences ($\Delta\Delta G$) associated with the mutations, shows that both forces are contributory.

Certain tricyclic amines containing a bulky exocyclic group such as ethopropazine are highly selective for BuChE. This selectivity appears to be entirely a result of steric hindrance. The position of the diethylaminoisopropyl side chain of ethopropazine overlaps with that of Y_{337} . The $Y_{337}A$ substitution in an AChE template yields a mutant which virtually replicates the binding affinity

of ethopropazine for BuChE [6]. Other participating side chains in the active center, the catalytic triad (E₃₃₄, H₄₄₇, S₂₀₃), in which the active center serine (S₂₀₃) attacks the carbonyl carbon, and the oxyanion hole (G₁₂₀, G₁₂₁, A₂₀₄) are invariant among the cholinesterases.

The third domain responsible for ligand specificity lies outside the active center residing at the lip of the gorge. This region contributes to the peripheral anionic site. Certain ligands, the prototype of which is propidium, bind to the peripheral site regulating catalysis in an allosteric fashion [6]. Fasciculin, a peptide toxin of 6,500 Da that is homologous to the three loop, short α -toxins, also binds to this site. Bisquaternary ligands in which the quaternary groups are separated by at least 14Å span between the peripheral site and active center. Site-specific mutagenesis shows that at least four residues encompass the peripheral site, W₂₈₆, Y₇₂, Y₁₂₄ and D₇₄. H₂₈₅ and Y₁₃₀ may also be involved. W₂₈₆, Y₇₂, Y₁₂₄ exist as R(A), Q, and N respectively in BuChE. Remarkably, substitution at only these three positions to the residues found in BuChE confers BuChE characteristics to this site where fasciculin, propidium and certain bisquaternary ligands bind with markedly lower affinities [17,20]. Some bisquaternary ligands and propidium likely bind in a different orientation on BuChE than AChE.

Perhaps the most interesting peripheral site ligand is fasciculin for it binds to AChE with a K_d of 2 pM and to BuChE with a K_d of 230 μ M. Partitioning of the free energy shows that substitutions of W₂₈₆R, Y₁₂₄Q, Y₇₂N can account for the entire specificity difference [11]. Hence this relatively large peptide residing at the lip of the gorge restricts substrate access and allosterically affects the alignment of residues in the active center gorge (Radic', Z., et al., submitted). Studies of expression and mutagenesis of the fasciculin molecule are underway. Some striking contrasts can be developed between fasciculin and the α -toxins. First, fasciculin does not bind to the main acetylcholine recognition site on AChE, whereas the α -toxins show a competitive interaction with the acetylcholine recognition site on the receptor. Second, glycosylation of the receptor confers α -toxin resistance while it plays no role in AChE-BuChE specificity differences. Third, a subunit interface is required for high affinity binding of the α -toxins but not for fasciculin.

Ligand Specificity at the Nicotinic Acetylcholine Receptor

The absence of a high resolution structure of the nAChR necessitates a different approach to the analysis of structure. Site-directed labeling with chemically reactive reagents, photoreactive reagents and natural conjugating toxins [12,13] have defined candidate residues involved in agonist and antagonist binding to the receptor. Reactive residues in the α -subunit include C₁₉₂, C₁₉₃, Y₉₃, Y₁₉₀, Y₁₉₈ and W₄₉ in the α -subunit and their roles in ligand specificity have been examined further by mutagenesis.

Inserting mutations at residues where sequence differences between species might be responsible for functional differences offers a second approach. This approach, for example, enabled us to define residues conferring resistance to snake α -toxins for the snake and mongoose nAChR [14]. By selecting sequence differences [15], we have determined that glycosylation signals at N₁₈₉ in snake and N₁₈₇ in mongoose are the primary substitutions necessary to confer resistance to the α -toxin. This interference from presumed steric hindrance is seen with the 6,800 Da α -toxins but not for the 1,500 Da α -conotoxin or the 500 Da lophotoxin. The observation that only glycosylation affects α -toxin binding differs from data acquired with isolated peptides and fusion proteins and likely reflects the necessity of a proper apposition of amino acid residues at the subunit interface in order to achieve ligand specificity.

The third approach relies on distinct sequences in the domains of the γ and δ subunits associating with the same face of α . A receptor model shows that the counterclockwise face of α will associate with the clockwise faces of γ and δ . These two interfaces form the two binding sites for agonists. When the dissociation constants at these sites differ, it becomes possible through γ , δ substitutions to define residues contributing to the interfacial binding sites [14]. This approach has been used to define the residues giving a 100-fold preference of the α, γ over the α, δ interface in *d*-tubocurarine binding [17]. An even more dramatic example is the 10^4 preference in α -conotoxin binding found for the α, δ over that of the α, γ interfaces [14].

A two subunit transfection of α and γ or α and δ yields dimeric receptor with appropriate ligand binding properties. For example, the difference in specificity seen for the α, γ in an intact pentameric receptor can be replicated in assembled dimers. However, the assembled dimers do not reach the cell surface. Three subunit transfections of $\alpha\beta\gamma$ or $\alpha\beta\delta$ yields pentameric $\alpha_2\beta\gamma_2$ and $\alpha_2\beta\delta_2$, which are expressed on the cell surface as does the native $\alpha_2\beta\gamma\delta$. Only the four subunit transfection, $\alpha\beta\gamma\delta$, gives the requisite cooperativity seen in the intact receptor [18]. Mutagenesis has also been employed to examine assembly of receptor subunits.

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MOLECULAR FORMS OF ACETYLCHOLINESTERASE: STRUCTURE AND INTERACTIONS

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Acetylcholinesterase exhibits a number of molecular variants, which arise from a combination of alternative splicing and post-translational modifications (Massoulié *et al.*, 1993). We will analyze the different steps leading to the mature enzyme forms and show that they can be individually regulated.

A The polymorphism of acetylcholinesterase: post-transcriptional and post-translational processes

1) Catalytic subunits of AChE generated by alternative splicing: R, H and T

In vertebrates, several catalytic subunits of acetylcholinesterase (AChE, EC 3.1.1.7) are generated from a single gene by alternative splicing (Sikorav *et al.*, 1988; Maulet *et al.*, 1990; Li *et al.*, 1991). The polypeptides consist of a signal peptide, a catalytic domain (535 amino acids in *Torpedo* AChE), and differ by C-terminal peptides encoded by alternative exons. Three types of coding sequences have been characterized in cDNAs.

The R ("Readthrough") transcripts retain the genomic sequence immediately downstream of the last common exon. This type of organization has been observed in *Torpedo*, murine and human cDNAs, but the deduced peptide sequence is highly variable in these three species (Sikorav *et al.*, 1988; Li *et al.*, 1991; Legay *et al.*, 1993b). The predicted R subunits have not yet been characterized *in vivo*, and their physiological significance is, in fact, problematic, since the other two types of subunits, H and T, fully account for all the molecular forms of AChE identified so far (Duval *et al.*, 1992a; Legay *et al.*, 1993a). The production of the R transcript does not, however, seem to represent a default in the splicing process because R transcripts constitute a significant proportion of the AChE transcripts in some tissues, particularly in murine embryonic liver and muscle (Legay *et al.*, 1993b), as well as in hematopoietic cells (Li *et al.*, 1991), and they are developmentally regulated, as discussed below.

The C-terminal peptide encoded by the H ("hydrophobic") transcript contains a cysteine residue involved in inter-subunit disulfide linkage and a signal peptide for cleavage/addition of a GPI anchor. It thus produces GPI-anchored dimers (amphiphilic forms of type D), which constitute the major form of AChE in *Torpedo* muscles, and are found on the surfaces of blood cells in mammals. In contrast, birds do not seem to possess H subunits: an analysis of the genomic sequence located between the common exon and the downstream T exon in quail did not reveal the presence of a potential alternative H exon (A. Anselmet, unpublished results), and in fact GPI-anchored AChE dimers have not been detected in birds, particularly on erythrocytes (S. Bon, unpublished results).

In the T ("tailed") transcript, the alternatively spliced exon encodes a 40-amino acid C-terminal peptide, which is retained in the mature enzyme. This T peptide contains a cysteine residue, located at position -4 from its C-terminus, which is involved in homologous or heterologous inter-subunit disulfide bonds. In mammalian and avian muscles and in nervous tissue, AChE is composed only of T subunits. These subunits, however, may be organized in a number of various molecular forms, ranging from monomers (G_1), homo-oligomeric dimers (G_2) and tetramers (G_4), to the hetero-oligomeric hydrophobic-tailed tetramers and collagen-tailed forms, which include up to twelve catalytic subunits (A_{12}). The monomers and dimers of T subunits interact with detergent micelles, under nondenaturing

conditions, and thus represent amphiphilic forms of type II, as opposed to GPI-anchored AChE (Bon *et al.*, 1991). The tetramers are mostly nonamphiphilic.

The amphiphilic properties of the monomers and dimers of T subunits, as well as the various homomeric and heteromeric quaternary associations in which they participate depend on the presence of the 40-amino acid T_C peptide, since truncated subunits, in which a stop codon had been introduced at the beginning of this peptide, only generated soluble monomers (Duval *et al.*, 1992a).

Active and inactive molecular forms of AChE

In birds, only T subunits appear to exist, so that the biosynthesis of AChE appears relatively straightforward. Analysis of AChE in chicken brain and muscle, however, demonstrated the presence of inactive AChE protein and showed that the acquisition of catalytic activity seems to be subject to specific control, at least in birds. It should be noted that an inactive component of AChE was not observed in rat (Brimijoin *et al.*, 1987). A comparison of the catalytic activity and immunoreactivity of AChE with specific monoclonal antibodies shows that different fractions from chicken tissues contain a variable proportion of inactive AChE protein (Chatel *et al.*, 1993a). We quantified the inactive AChE component by two-site immunoradiometric assays (Chatel *et al.*, 1994): AChE was specifically retained by an immobilized antibody and quantified by a second radiolabelled antibody. We used two different capture antibodies: the C-6 antibody, which recognizes the totality of the AChE protein, and the C-131 antibody, which is conformation-dependent and preferentially recognizes active AChE (Chatel *et al.*, 1993b). The second antibody, ¹²⁵I-C-54, was the same in both cases, so that the results could be directly compared.

An analysis of gradient fractions, using C-6/C-54 and C-131/C-54 immunoassays as well as activity determination, shows that the A₁₂ and G₄ forms are composed exclusively of active subunits, while inactive molecules co-sediment with the active G₂ and G₁ forms. Both active and inactive G₂ and G₁ forms are amphiphilic, as indicated by the influence of detergents on their sedimentation coefficients and Stokes radii. In addition, the inactive molecules appeared to react with an organophosphate inhibitor, like the active enzyme. It is therefore likely that even the "inactive" subunits possess a catalytic triad and, perhaps, a low catalytic activity.

Thus, AChE subunits seem capable of assuming distinct conformations which, although very similar, differ in their catalytic activity, in their reactivity with the monoclonal antibody C-131, and in their capacity to form tetramers. As discussed below, the proportions of the two conformations vary with development and cell differentiation, suggesting that the cells actively control the folding of the protein and the acquisition of catalytic activity.

2) Amphiphilic character of type II AChE forms: the T subunits of acetylcholinesterase possess a C-terminal amphiphilic α -helix

Since truncated T subunits, lacking all but the first four aminoacids of the C-terminal T_C peptide, generate only nonamphiphilic monomers, it is clear that it is responsible for the hydrophobic properties of the type II amphiphilic forms, as mentioned above. Because T_C does not contain a classical hydrophobic sequence, we examined whether this property depended on the peptide itself, or whether it required some post-translational modification (Bon *et al.*, in preparation). For this purpose, we covalently coupled the chemically synthesized peptide to a nonamphiphilic form of AChE, the lytic tetramers derived from *Electrophorus* collagen-tailed forms. The resulting enzyme was then able to bind detergent micelles, demonstrating that the peptide itself is sufficient to confer this property.

We showed that the peptide could be modelled as an amphiphilic α -helix, exposing hydrophobic aminoacids on one sector, including three tryptophan residues, equally spaced by six intervening residues. Such an organization would explain the hydrophobic interactions. Fluorescence spectroscopy and circular dichroism confirmed that the peptide assumes an α -helical conformation, which is stabilized in the presence of detergent micelles and phospholipids. The tryptophan residues are buried under these conditions. The importance of the tryptophan residues for the amphiphilic character of monomers and dimers of T subunits will be verified by site-directed mutagenesis.

3] Collagen-tailed forms: association of T subunits and collagenic tail subunits

In the collagen-tailed forms, tetramers of T subunits are disulfide-linked to each strand of a collagenic triple helix. In addition to their original structure, collagen-tailed forms are particularly interesting because they are restricted to differentiated nerve and muscle cells, while other forms of AChE, containing the same T subunits, are more widely distributed. Collagen-tailed forms are tethered through ionic interactions with glycosaminoglycans in the synaptic basal lamina of neuromuscular junctions. The presence of these forms, their synaptic localization, the ratio of molecules, containing one, two or three tetramers (A_4 , A_8 , A_{12}), are controlled by innervation and by the fast or slow mode of muscle activity (Lømo *et al.*, 1985; Vallette and Massoulié, 1991). They thus constitute a sensitive index of muscle differentiation. Moreover, their physiological importance is dramatically demonstrated in a human congenital myasthenia associated with end-plate AChE deficiency (CMEAD), in which these forms are specifically lacking (Hutchinson *et al.*, 1993).

A cDNA encoding the collagenic tail subunit, Q, was cloned from *Torpedo* electric organ. When co-expressed in transfected COS cells, it assembled with T subunits to form collagen-tailed AChE (Krejci *et al.*, 1991). The complementarity between catalytic and structural subunits is remarkably conserved throughout vertebrates, since collagenic subunits generate hybrid collagen-tailed forms with T subunits of rat and of human AChE (Legay *et al.*, 1993a; Camp *et al.*, in preparation), as well as with the homologous T subunits of human butyrylcholinesterase (BChE) (S. Bon, unpublished results). The pivotal role of the T_C peptides in these quaternary interactions is quite consistent with the fact that they are more conserved among vertebrate AChEs and BChEs than the catalytic domains.

We showed that the tetramers were attached to the N-terminal non-collagenic domain of Q, rather than to the C-terminal domain: mild collagenase treatment removed the latter domain, as indicated by specific antibodies to this domain, without disrupting the association of T tetramers with the collagenic fragment. We further showed that the N-terminal domain was sufficient to bind a T tetramer, by constructing a chimeric protein in which this domain was fused to the GPI addition signal of the H subunit: we thus obtained GPI-anchored tetramers, which do not exist in nature (Duval *et al.*, 1992b).

B Regulation of the biosynthesis of AChE forms

1] Developmental regulation and focalization of acetylcholinesterase mRNA in the mouse diaphragm

We used *in situ* hybridization and the polymerase chain reaction (PCR) to analyze the nature and distribution of AChE mRNAs along the myofibers of the mouse diaphragm, during development (Legay *et al.*, in preparation). While adult muscle contains only the T transcript, expressed by junctional nuclei (Jasmin *et al.*, 1993), we were surprised to find the three types of AChE mRNA from embryonic day 13 (E_{13}) up to birth: T is already predominant at E_{13} , R represents a few percent

of mRNA and H is detectable at still lower levels. This is the first report of the existence of the R transcript in muscle. We obtained similar results with the C2 myogenic cell line, both as myoblasts and differentiated myotubes.

As early as E₁₃, T mRNAs preferentially accumulate in the midline of the diaphragm, where the first neuromuscular contacts are forming. Thus, the selective compartmentalization of AChE mRNA seems to concern only one of the splice variants.

2] *Evolution of acetylcholinesterase transcripts and molecular forms during development in the central nervous system of the quail.*

The expression and biosynthesis of AChE is relatively simple in the nervous system of the quail, since there is only a single type of transcript, encoding T subunits, and these subunits only generate monomers, dimers and tetramers, but no collagen-tailed forms. We studied AChE mRNAs and AChE molecular forms in the cerebellum, optic lobes and neuroretina of the quail at different stages of development, from embryonic day 10 (E10) to the adult (Anselmet *et al.*, 1994). We observed developmental variations in:

i) production of multiple mRNA species (4.5 kb, 5.3 kb and 6 kb) The large transcripts present distinct temporal patterns and disappear in the adult, which possesses only the 4.5 kb mRNA; these changes might be related to the stability of mRNAs. The levels of mRNA and AChE are not correlated in the three regions, especially at the adult stage.

ii) production of active and inactive AChE molecules. The proportion of inactive AChE was found to be markedly higher at the hatching period (E16) than either at earlier stages (E10 and E13) or in the adult.

iii) proportions of tetrameric G₄, dimeric G₂ and monomeric G₁ forms. The G₄ form is predominant already at E10, and in the adult its proportion reaches 80% of the activity in the cerebellum and optic lobes, 65-70% in the neuroretina.

iv) production of amphiphilic and nonamphiphilic AChE forms. The G₄ form is largely nonamphiphilic in embryonic tissues, but the proportion of the amphiphilic component progressively increases with development.

Thus the different processing and maturation steps appear to be regulated in an independent manner and potentially correspond to physiologically adaptative mechanisms. A similar conclusion was reached in the case of the chicken (Chatel *et al.*, 1994).

3] *Regulation of the biosynthesis of hetero-oligomeric forms*

The fact that collagen-tailed forms readily assemble in COS cells suggests that their production simply reflects a restricted expression of the collagenic Q subunits in differentiated nerve and muscle cells. It is interesting that, in muscles, these forms appear to be controlled by innervation, but in a manner that differs markedly between species.

The membrane-bound G₄ form that constitutes the major form of AChE in mammalian brain is also heteromeric: a 20 kDa hydrophobic subunit is disulfide linked to one of the dimers, through the same C-terminal cysteine of the T subunits as attaches to the collagen tail. It thus seems to result from very similar quaternary interactions, and its production may also be limited by the availability of the structural subunit. It is interesting that this form is specifically increased or decreased in rat muscles, depending on exercise, while the level of the other AChE

forms, including the collagen-tailed forms, is not modified (Gisiger *et al.*, 1988) and that its level is altered in muscle dystrophy (Gisiger *et al.*, 1991), e.g. in the mdx mouse (Oliver *et al.*, 1992). Thus, each type of AChE seems to respond to a specific physiological need.

The cloning and characterization of the associated anchoring subunits, in mammals, should make it possible to analyze this expression, which may be an interesting index of cellular differentiation, and to explore the functions of the heteromeric forms. In addition, the genetic defect that causes CMEAD is likely to reside in the gene or genes encoding these collagenic subunits, since no abnormality was found in the AChE gene from a CMEAD patient, and the derived AChE T subunit was able to combine with Q subunits, forming collagen-tailed molecules exactly like in a normal control (Camp *et al.*, in preparation).

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3-D Structure of Acetylcholinesterase and Complexes of it with Anticholinesterase Agents

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The principal biological role of acetylcholinesterase (AChE, acetylcholine hydrolase, EC 3.1.1.7) is to terminate signal transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter, acetylcholine (ACh)^{1, 2}. In keeping with this requirement, AChE possesses a remarkably high specific activity, especially for a serine hydrolase³, functioning at a rate approaching that of a diffusion-controlled reaction⁴. Early kinetic studies indicated that the active site of AChE consists of two subsites, the 'esteratic' and 'anionic' subsites, corresponding to the catalytic machinery and the choline-binding pocket, respectively⁵. A second, 'peripheral', anionic site exists, so named because it appears to be distant from the active site⁶. The recent elucidation of the three-dimensional structure of Torpedo AChE⁷ has served to confirm these earlier studies, and has shown that AChE contains a catalytic triad similar to that present in other serine hydrolases⁸. Unexpectedly, it has also revealed that this triad is located near the bottom of a deep and narrow cavity, ca. 20 Å deep, which has been named the 'active-site gorge'. The cavity is lined by the rings of fourteen aromatic residues which are conserved in the AChE sequences published so far⁹. Much of the subsequent research on structure-function relationships in AChE has been concerned with the functional significance of the gorge and with the role of the aromatic rings which account for more than 50% of its surface area¹⁰. Thus, structural evidence^{7, 10}, as well as evidence obtained by modelling¹¹, by chemical modification¹²⁻¹⁴, and by site-directed mutagenesis^{11, 15-18}, all point to important roles for certain of these conserved aromatic residues in both the esteratic and 'anionic' subsites of the active site, and in the 'peripheral' anionic site. In order to study experimentally this interaction, in detail, we soaked into crystals of AChE a series of different inhibitors, including the competitive inhibitor edrophonium (EDR), tacrine (THA), decamethonium (DECA)¹⁴ & the transition-state analog (N,N,N-trimethylammonio)trifluoroacetophenone (TFK), and determined their 3-D structures.

This enzyme generates a strong electrostatic field that can attract the cationic substrate, acetylcholine (ACh), to the active site¹⁹. However, the long and narrow active site gorge appears inconsistent with the enzyme's high catalytic rate (turnover number 20,000/sec). A molecular dynamics simulation of AChE, in water, which has revealed a transient opening of a short channel, large enough to pass a water molecule through a thin wall of the active site near W84. This suggests that substrate, products, and/or solvent, could move through a *back door*, in addition to the route suggested by the crystallographic structure^{10, 20}. These calculations show a strong electrostatic field at the *back door*, oriented to attract cations, such as the substrate & one of the reaction products, choline, and to repel anions, such as the other reaction product, acetate. These studies suggest a novel hypothesis that thermal motion of an enzyme may cause it to have multiple routes of access to the active site.

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*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
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NOTES

NOTES

THURSDAY, NOVEMBER 9, 1994

(AFTERNOON)

CHOLINESTERASE II

Chair: Dr. B.P. Doctor; Co-Chair: Dr. A. Eldefrawi

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|-------|----------------|--|
| 14:30 | Rotundo, R.L. | Compartmentalized Regulation of Acetylcholinesterase Expression in Skeletal Muscle |
| 15:00 | Randall, W.R. | Neural Regulation of Acetylcholinesterase Gene Expression |
| 15:30 | Soreq, H. | Engineering Cholinergic Synapses Through Overexpression of Human Cholinesterase in <i>Xenopus</i> Tadpoles and Transgenic Mice |
| 16:00 | Rosenberry, T. | Genetic Analysis of Glycoinositol Phospholipid (GPI) Anchor Function in <i>Drosophila</i> Acetylcholinesterase |

16:30 FINAL REMARKS

E. X. Albuquerque, F. Hucho, R. Rahamimoff, I. Silman

COMPARTMENTALIZED REGULATION OF ACETYLCHOLINESTERASE EXPRESSION IN SKELETAL MUSCLE

Rotundo, R.L., Rossi, S.R., Godinho, R.O., Vazquez, A.E., and Trivedi, B.

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The establishment of polarity in eukaryotic cells and the organization of specialized membrane domains requires that all the necessary molecular machinery be transported to, and selectively retained at, the appropriate regions of the plasma membrane. For most eukaryotic cells a single nucleus is the rule, and cell polarity is established through interactions with neighboring cells and with the extracellular matrix upon which they rest. Membrane and secreted proteins are synthesized on the rough endoplasmic reticulum, processed in the Golgi, and are sorted upon exit through the trans-Golgi network of cisternae and vesicular structures. Subsets of transport vesicles can then carry their cargo, either constitutively or for stimulus-coupled exocytosis, which are then externalized at the apical or basolateral membranes. In contrast, multinucleated skeletal muscle fibers appeared to behave as "bags" of nuclei, sometimes numbering in the many hundreds or even thousands per fiber, which were believed to be expressing a relatively homogenous sub-population of genes. Clearly these earlier views were somewhat oversimplified, and studies from many different laboratories over the past few years have demonstrated that skeletal muscle fibers are compartmentalized with respect to the expression of several different genes, the most dramatic examples being the localized expression of nicotinic acetylcholine receptor (nAChR) subunits and acetylcholinesterase (AChE) at the neuromuscular junction.

Compartmentalization of AChE mRNA and Protein Expression in Adult Skeletal Muscle:

Acetylcholinesterase was the first molecule shown to be highly concentrated at synaptic sites, demonstrated by Couteaux and Nachmansson in 1938 a manometric enzyme assay on frozen sections of muscle. Since that time AChE has been one of the important markers for studies on nerve-muscle interactions and regulation of synaptic components. Transcripts encoding AChE are also highly concentrated at sites of nerve-muscle contact. Using quantitative reverse transcriptase PCR to determine AChE mRNA levels in innervated and noninnervated segments of single skeletal muscle fibers, Jasmin et al. (1993) found that the transcripts predominated at the neuromuscular junction, whereas they were almost undetectable in the adjacent non-innervated regions. Analysis of enzyme activity in a parallel sample of single neuromuscular junctions showed that AChE was concentrated approximately 30-fold at the sites of innervation. However, one puzzling observation was that AChE transcripts were not detected at all neuromuscular synapses. In fact approximately half the junctions sampled contained no detectable AChE mRNA even though they expressed the same levels of the muscle-specific α -actin mRNA as their positive counterparts. In addition, an AChE primer sequence-specific internal standard matched to the concentrations of the endogenous mRNA was easily detected. These observations suggest the interesting possibility that not all synapses are expressing the relevant transcripts at the same time, leading to the possible model of intermittent versus constitutive regulation of this important synaptic component.

AChE Expression is Compartmentalized in Myotubes:

Compartmentalized expression of AChE does not appear to be an exclusive property of the

differentiated skeletal muscle fiber *in vivo*, but rather an intrinsic property of the organization of multinucleated muscle fibers in general. Evidence from several laboratories has now provided strong evidence that different classes of proteins, including components of the myofibrillar apparatus, proteins localized to intracellular organelles, and several membrane bound and extracellular matrix proteins are organized and localized in the vicinity of the nuclei encoding them. In fact, even viral proteins expressed in multinucleated skeletal muscle fibers are locally targeted to the overlying sarcolemma.

In the case of AChE, the catalytic subunits are locally translated on the rough endoplasmic reticulum surrounding the nucleus where the mRNA was transcribed (Rotundo, 1990). Not only are the catalytic subunits locally assembled, they are transported and attached to the extracellular matrix overlying the nucleus of origin (Rossi and Rotundo, 1993). These studies show that not only is protein synthesis, processing, and transport highly localized, they also indicate that each nucleus is capable of organizing the surface plasma membrane and extracellular matrix within a defined "sphere of influence". This sphere of influence is herein referred to as cell surface nuclear domains.

Evidence for Local Control of AChE Expression in Tissue-Cultured Muscle:

The expression of AChE in skeletal muscle is regulated by both electromechanical activity of the muscle fibers and by information provided by the nerve. Although the nerve provides positional information for the deposition of AChE at sites of nerve-muscle contact, through the deposition of specific molecules such as Agrin on the synaptic basal lamina, the nerve also provides information for the moment to moment regulation of AChE transcription and translation via release of the neurotransmitter acetylcholine. Thus the frequency, as well as intensity, of membrane depolarization also plays a role in AChE regulation. In culture, differences in membrane depolarization can be mimicked by agents which either increase or prevent membrane depolarization, such as veratridine (Ver), scorpion toxin (ScTx), and tetrodotoxin (TTX). Sodium channel agonists, such as Ver and ScTx act to increase AChE expression, and assembly of the collagen-tailed AChE form, whereas agents which block sodium channels, such as TTX, act to suppress AChE expression and assembly of asymmetric AChE. These effects can be measured biochemically, by assays of enzyme activity, or by immunohistochemical localization of the AChE molecules expressed on the cell surface.

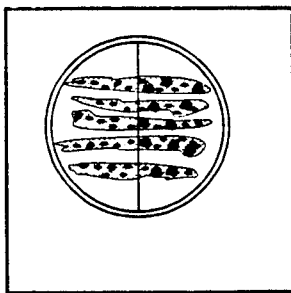


Figure 1: Parallel myotube culture microchamber: Quail embryo myoblasts plated on scratched collagen-coated glass coverslips mounted in a retainer ring are grown in normal medium until day five when fusion and differentiation is complete and the myotubes become spontaneously contractile. A partition made from a piece of glass coverslip and coated with a thin layer of Dow-Corning silicone grease is then lowered over the fibers. The mean fiber length is about 1500 μm and the coverslip width is about 120 μm . To one side of the chamber is added the test compound while the other side is the control. Cell surface AChE clusters and distribution are quantitated only on those fibers which extend for several hundred μm on either side of the partition.

To determine whether signals from the overlying regions of the plasma membrane were responsible for locally regulating AChE expression, tissue-culture microchambers were constructed so as to allow local application of membrane-impermeant drugs which either increased or decreased AChE expression (Figure 1). Local application of the sodium channel agonists or antagonists, such as ScTx or TTX, to portions of the plasma membrane resulted in local increases or decrease in

AChE expression. These studies indicate that each nucleus responds specifically to signals generated on the overlying region of the plasma membrane, and furthermore, suggests the interesting possibility that each nucleus in the skeletal muscle fiber is under local control by its environment.

Localization of Signal Transduction in Myotubes:

Tissue cultured quail myotubes express a population of muscarinic receptors linked to phosphatidylinositol turnover (Godinho and Rotundo, 1994). Stimulation of myotubes with oxotremorine-M or carbachol in the presence of nicotinic receptor antagonists stimulates production of diacylglycerol (DAG), whereas incubation with nicotine or carbachol in the presence of atropine or QNB has no effect. Autoradiographic localization of sites of DAG production using ^3H -cytidine in the presence of LiCl_2 reveals that formation of DAG is highly localized to a small region overlying the myotube nuclei. In nonstimulated myotubes, only a very small fraction of the nuclei show accumulations of DAG in the presence of LiCl_2 alone, whereas in stimulated cultures the fraction of positive nuclei approaches 100%. These studies indicate that even signal transduction systems linking changes in membrane activity to changes in gene and protein expression can be highly compartmentalized in skeletal muscle fibers.

Second Messenger Systems and AChE Regulation:

Although the complete pathways involved in AChE regulation are still unknown, the involvement of several second messenger systems has already been identified. Membrane depolarization has been shown by several laboratories to be an important regulatory event, but the molecular mechanisms downstream of the initial event remain to be determined. Influx of Ca^{2+} has been implicated as a mediator, and these observations are supported by studies showing that nifedipine, a potent inhibitor of L-type Ca^{2+} channels, reduces asymmetric AChE levels in a manner similar to TTX. Activation of protein kinase C via phorbol esters can mimic, at least in part, the effects of membrane depolarization with respect to increasing levels of the asymmetric AChE form, even in TTX-paralyzed myotubes. In contrast, agents that increase cAMP levels exert a rapid inhibitory effect on asymmetric AChE expression with little or no effect on total AChE synthesis. However, agents that completely inhibit protein kinase C activity, such as chelerythrine, reduce cellular levels of AChE by 80-90%. These effects appear to be at the level of AChE translation since they occur rapidly in the myotubes. It thus appears that a combination of several second messenger systems, at least one of which is directly linked to membrane depolarization, are involved in both stimulating and repressing AChE expression. A detailed localization of these second messenger systems in adult skeletal muscle fibers will provide important information concerning the extent of compartmentalization of the signal transduction systems at the neuromuscular junction.

Summary and Conclusions:

Although multinucleated skeletal muscle fibers are not "polarized" in the traditional sense of the term, such as the clearly defined apical and basolateral membrane domains of specialized epithelia and neurons, the highly localized patterns of sorting, organization, and regulation of many membrane, secreted, and cytoskeletal proteins observed in these complex cells clearly indicates a high degree of polarized function. A corollary to the establishment of specialized membrane domains in polarized epithelia is the establishment of localized signal transduction systems capable of regulating a variety of intracellular functions including gene expression.

In concert with the compartmentalized expression of proteins in skeletal muscle, in vivo and in culture, it now appears that the signal transduction systems themselves are also compartmentalized. In our experiments, signals generated locally at the level of the plasma membrane are shown to regulate expression of AChE encoded by the nuclei beneath the sites of signal generation. Thus each nucleus in the multinucleated fiber appears to receive information locally, and can thus act independently. These observations are particularly pertinent to the regulation of genes encoding synapse-specific proteins since the extent to which they are expressed (or repressed) would depend on the types of signals generated (or not generated) at the overlying regions of the sarcolemma. These signals can be generated directly via substances released from the nerve terminal, or indirectly as a consequence of membrane depolarization and/or muscle contraction. The specific subsets of genes expressed would therefore be dependent upon the signaling milieu in which an individual nucleus was located, and the localization of specific signal transduction systems would depend, at least in part, upon the types of local cell-cell and cell-matrix interactions.

Acknowledgments:

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NEURAL REGULATION OF ACETYLCHOLINESTERASE GENE EXPRESSION. Randall, W.R.
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The correct postsynaptic organization of acetylcholinesterase (AChE) at the neuromuscular junction requires the initiation and maintenance of functional innervation. The molecular mechanisms by which the genes encoding the catalytic and structural subunits of the enzyme are regulated by the nerve are largely unknown. The initiation of nerve contact during muscle development causes the density of AChE to concentrate at newly formed synapses while the extrajunctional levels decline as the muscle matures. The reversal of this phenomenon in large part is mimicked by denervation. In the chicken, denervation induces an increase in extrajunctional AChE activity which is suppressed by electrical activity. The coordinate regulation of AChE and subunits of the nicotinic nAChR during the onset of innervation and in response to denervation suggests that the underlying mechanisms may be similar in many instances. Our objectives are to determine the genetic elements of the catalytic and structural subunits of AChE necessary for the synaptic control of its expression.

Our initial studies focused on the expression of AChE transcripts following denervation of the muscle to serve as a basis for the studies of the neural regulation of AChE gene activity. Denervation induces an increase in the transcript level of AChE in the fast twitch posterior latissimus dorsi (PLD) 10-20 fold above the innervated contralateral muscle (Fig. 1, PLD). The response reaches maximal levels by six days and maintains the high level while the muscle remains denervated. Similarly, the slow tonic anterior latissimus dorsi (ALD) responds to denervation but with a more modest increase (~4-fold) in AChE transcript levels (Fig 1, ALD). This may reflect the fact that the multiply innervated muscle expresses more transcript in the innervated state than the PLD. The collagen-tailed form of AChE, normally concentrated at the synapse, disappears upon denervation (3). Similarly, transcripts encoding the collagen-like Q- subunit of AChE decline by three days after denervation and are not detectable by six days.

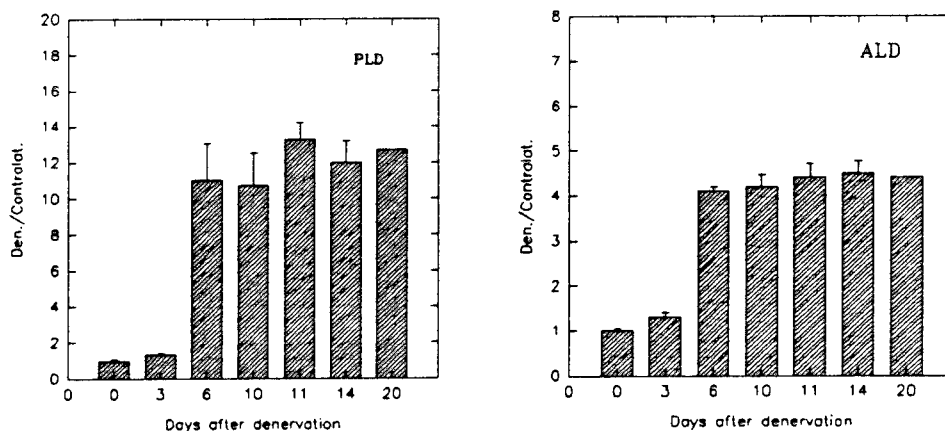


Figure 1. AChE transcript levels of the PLD and ALD after denervation. Muscles of adult chickens were denervated for the times indicated by sectioning of the brachial plexus and the RNA from the muscles ($n=4 \pm s.e.$) was subsequently analyzed for AChE transcripts by quantitative Northern and dot blot analysis. D/C is the AChE transcript ratio of the denervated to contralateral muscles.

The observed increase in AChE transcript raises the question of whether it is due

to an increase in the synthesis of nascent transcripts or to an increase in their stability or a combination of both. To assess whether increased synthesis of transcripts contributed to the change, transcript elongation by nuclear run-on of denervated and contralateral innervated leg muscle was examined (Table 1.)

Probe	Innervated (Vol.)	Denervated (Vol.)	Ratio (Den./In.)
M13	3290	2340	0.71
AChE antisense	8409	24844	2.95
AChE sense	2574	2749	1.06
MyoD antisense	5375	6616	1.23

Table 1. Transcript elongation of denervated and contralateral chick leg muscle.

Denervations were performed on the lower leg muscles of 2-day-old chicks by cutting the sciatic and peroneal nerves. Nuclei were isolated and extracts prepared from the dissected muscles 2 days after denervation and the elongation assays were performed using 250 uCi [³²P]UTP per extract. Nascent [³²P]-labeled transcripts were separated from unincorporated label and hybridized to 10 µg of single stranded DNA probes cloned into M13 and immobilized on nitrocellulose. Quantitation of blots by PhosphorImaging was performed and mean values from two experiments, corrected for background, are shown. The probe for AChE was a 750 bp fragment containing internal coding region of the transcript.

These data indicate that nascent AChE transcripts are synthesized at levels approximately 3-fold higher in the denervated muscle than in the innervated muscle. Hybridization was specific to the antisense strand; background levels of hybridization were observed for the M13 vector alone and the AChE sense strand. The transcription rate of chick MyoD showed little change with denervation as previously reported (2). These data suggest that part of the observed increase in transcript level for the AChE catalytic subunit results from an increase in its rate of synthesis.

Gene and Transcript Structure of Chicken AChE. The characterization of the gene and transcripts encoding chicken AChE was taken as the next step in identifying the genetic components required for neural regulation. The chicken AChE gene shows a highly conserved exon/intron pattern compared with other vertebrate cholinesterases (Fig. 2, above). One distinguishing feature is the lack of the *H* exon encoding the alternatively spliced C-terminal that gives rise to the GPI anchored form of AChE in other vertebrates. The strongest evidence for this observation was provided by examining the complete DNA sequence of the intron region spanning exons 4 and 5. No coding region was found in any reading frame that contained a consensus sequence necessary for GPI modification.

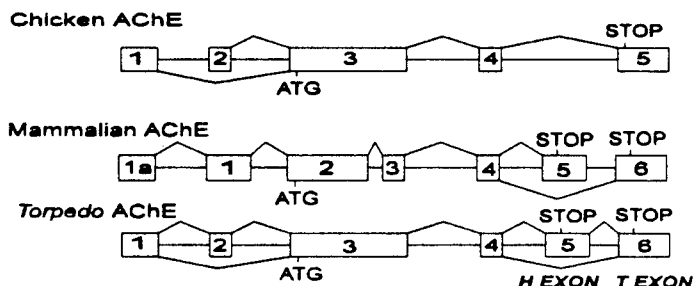


Figure 2. Gene structure of vertebrate AChEs. Exons are in boxes with angled connecting lines representing splice sites.

In addition, RNA from the hemopoetic tissues in the chicken (liver, spleen, bone marrow, blood) showed no detectable hybridization to probes from the intron region spanning exons

4 to 5, consistent with the known observation that chicken erythrocytes lack AChE activity.

A complete cDNA sequence of chicken AChE spans 4.535 kb and shows amino acid homology to AChE from other vertebrate species (1). A single polyadenylation signal is found at the 3' end and only one exon encoding 3' untranslated sequence is present in genomic clones. Probes containing sequence in exon 5 only hybridize with all AChE transcripts detected on Northern blots indicating that there is no alternative splicing at the 3' end of the chicken AChE primary transcript. Northern blot analysis of embryonic chicken pectoral muscle with probes encoding the catalytic T subunit of AChE reveal at least three major transcripts of ~4.5, 5.2 and 5.8 kb. Only two transcripts are found in RNA from adult brain and muscle, however. The transcripts are generated through alternative use of 5' untranslated regions. A 400 bp fragment of the 5' end of one class hybridizes to only the 5.2 and 5.8 kb transcripts in Northern blots of 13-day embryonic muscle. The second class of cDNAs extend 20 bp past the splice acceptor site in exon 3 and is sufficient in length to account for the 4.5 kb transcript. A 20 base oligonucleotide probe containing the sequence 5' of the splice site hybridizes with only the 4.5 kb transcript on a northern blot of 13-day embryonic chick muscle. These data suggest that at least two exons containing 5' untranslated sequence are alternatively spliced onto common sequence containing the complete open reading frame and 3' untranslated region of the AChE transcript. Genomic sequence flanking the 5' cap site of the 5.2 kb transcript have been established by primer extension and RNase protection assays and a fragment extending approximately 2 kb upstream of the cap site has been used in a reporter construction with nuclear targeted β -galactosidase (nlacZ) to examine its activity after DNA injection into muscles *in vivo*. The 2 kb fragment is sufficient to confer specificity of β -gal expression to synaptic nuclei in normal innervated leg muscle. In contrast, the specificity is lost in muscles that have been denervated for 5-days after the DNA injection.

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Running Title: Transgenic expression of
synaptic cholinesterases

ENGINEERING CHOLINERGIC SYNAPSES THROUGH OVEREXPRESSION OF HUMAN
CHOLINESTERASES IN XENOPUS TADPOLES AND TRANSGENIC MICE

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Defective cholinergic synapses have been associated with several neurodegenerative disorders of the central (Wurtman, 1992) and peripheral nervous system (Harding, 1992), suggesting that experimental changes in cholinergic neurotransmission can serve to study the relationship between cholinergic metabolism and synapse development and properties. To this end, we established transgenic models for overexpressing human cholinesterases (ChEs) (Soreq and Zakut, 1993) in neuromuscular junctions of transiently transgenic Xenopus laevis tadpoles and in brain of stably transgenic adult mice. ChE overexpression should create a local deficit of acetylcholine at the "engineered" synapses. This, in turn, was found to initiate feedback responses which shed new light on the role of cholinergic neurotransmission in regulating synaptic structure and function.

Two genes in humans encode ChEs. These are designated ACHE and BCHE and encode acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), respectively. We isolated these two genes, revealed the primary sequence of their closely related enzyme products and mapped them by in situ hybridization to the distinct chromosomal positions of 7q22 (ACHE) and 3q26 (BCHE) (Soreq and Zakut, 1993). Several point mutations abundant in the Israeli population were discovered in the BCHE gene (Ehrlich et al., 1994). A complex pattern of alternative splicing, unique to the human ACHE gene, was found to modify the 3'-end of AChE mRNAs in 3 different ways (Karpel et al., 1994). This, in turn, changes the C-terminus of the AChE protein and its anchoring potential. It was therefore of interest to determine whether any or all of these AChE and BuChE variants would be correctly targeted to synapses and examine the outcome of such synaptic targeting.

To prepare and characterize the DNA vectors required for studying the biological functions of the human ChE variants, we first employed microinjected oocytes of Xenopus laevis. Of the natural allelic variants of the BCHE gene, the common allelic substitution of aspartate at position 70 in BuChE by glycine created an enzyme incapable of hydrolyzing succinylcholine. This structure-function relationship unraveled the molecular basis for the well known clinical syndrome of "succinylcholine apnea", where patients subjected to muscle relaxation by succinylcholine experience delayed post-surgical recovery of independent breathing (Neville et al., 1992). To examine the peptide domain harboring Asp70 for its role

in determining the wide spectrum of substrate and inhibitor interactions in ChEs, we replaced by PCR engineering a 78 amino acid peptide including Asp70 in BuChE with the corresponding peptide from AChE. The resultant catalytically active chimera carried 12 non-conserved amino acid substitutions at the gorge lining domain and acquired many of the properties distinguishing AChE from BuChE with respect to inhibitor sensitivities (Loewenstein et al., 1993). A population diversity study then revealed 11% heterozygotes for Asp/Gly 70 among Israelies (Ehrlich et al., 1994). This predicted that variabilities dependent on population diversity may be observed in individual responses toward anti-AChE drugs employed in Alzheimer's therapy (Watkins et al., 1994).

Once structure-function relationships in the human cholinesterase variants were determined, we moved on to study the potential involvement of these enzymes in synapse development and functioning through their transgenic overexpression in *in vivo* milieus. To this end, DNA and/or mRNA sequences encoding for human BuChE or the brain and muscle form of human AChE that includes the alternative 3' exon E6 (E₆ AChE) were first microinjected into fertilized *Xenopus* eggs. This induced a several-days-long accumulation of the heterologous human enzymes, detected by cytochemical staining in neuromuscular junctions subjected to electron microscopy (Seidman et al., 1994). No major differences were observed in gross development or movement of these transiently transgenic tadpoles. However E₆AChE enlarged by up to 4-fold, within 2 days, the post-synaptic length of neuromuscular junctions in which this transgenic enzyme accumulated as compared with control junctions. Moreover, a double content of nicotinic acetylcholine receptors was measured in these tadpoles (Shapira et al., 1994), demonstrating that the transgenic overexpression of human AChE affected the level(s) of other cholinergic key protein(s). Interestingly, the DNA vector encoding the alternative E₅AChE did not affect synapse size and/or staining pattern, suggesting that this 3'-exon is primarily involved in determining enzyme localization (Seidman et al., in preparation).

To investigate the effect of AChE overexpression over cholinergic synapses in the mammalian brain, we placed the human AChE coding sequence under control of the human AChE promoter (Ben Aziz et al., 1993) and stably introduced it into the mouse genome according to techniques previously established for the BCHE gene (Beeri et al., 1994). Within the adult mouse brain, human E₆AChE was faithfully overexpressed in cholinergic brain neurons. Activity measurements revealed 2-fold increased AChE levels in frontal brain as compared with controls (Soreq et al., 1994). Using a species-specific mAb, multimeric assembly of this human AChE was observed by sucrose gradient centrifugations to be identical to that of the mouse brain enzyme. Moreover, *In situ* hybridization revealed transcription patterns resembling those of the host mouse gene. Here again, no gross changes were apparent in normal development and behavior of these mice. However, homozygous transgenic mice displayed significant resistance to the transient decrease in body temperature caused by both the anti-ChE Diisopropylfluorophosphate and the muscarinic inhibitor oxotremorine. This suggested increased levels of muscarinic acetylcholine receptors and modified cholinergic transmission under AChE overexpression in the mammalian brain.

The Cross-species tolerance of excessive cholinesterase activities which was observed in our studies suggests the existence of adjustment mechanism(s) which may restore balanced cholinergic functioning under conditions of imbalance. Our findings suggest the use of Xenopus tadpoles to search for the molecular basis of such mechanisms in early vertebrate embryogenesis. Furthermore, the transgenic mouse model can shed new light on the adjustment mechanisms operating in the adult mammalian brain.

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GENETIC ANALYSIS OF GLYCOINOSITOL PHOSPHOLIPID (GPI) ANCHOR FUNCTION IN *DROSOPHILA* ACETYLCHOLINESTERASE

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The covalent modification of proteins by glycoinositol phospholipids (GPIs) is a general mechanism by which cells from a large variety of eukaryotic organisms anchor certain proteins on the outer surface of the plasma membrane. Detailed biochemical studies have led to the elucidation of the structures of GPI anchors and their biosynthetic pathways (reviewed by Englund, 1993). The structures consist of a characteristic core glycan: EthN-P-6Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6-inositol phospholipid. Substituents branch from this core glycan to varying extents in different species. However, the functional roles of GPI anchors have remained elusive. Since the core glycan is highly conserved from protozoans to mammals and because many proteins have both GPI-anchored and transmembrane forms, it seems likely that GPIs have functions in addition to simply anchoring proteins on cell surfaces. An early hypothesis was that GPI-anchoring could be a means of regulating cell surface expression of a protein through GPI-specific phospholipases. Two phospholipases, a trypanosome phospholipase C (PLC) and a mammalian serum phospholipase D, are specific to inositol phospholipid linkages in GPIs. However, only scant evidence currently supports a cell surface function associated with such phospholipases, and the lifetimes of most GPI-linked proteins on the cell surface appear to be unaffected by phospholipase cleavage. Other indirect evidence of a functional role came from cell biological studies showing that a GPI acts as an apical, and possibly axonal, sorting signal in polarized epithelial cells and some mammalian neurons, respectively (see Dotti et al., 1991). A biochemical basis of such a role was suggested by observations that GPIs appear to associate with glycosphingolipids and cholesterol in membrane microdomains during transport to the apical surface of MDCK cells, that these microdomains can be isolated as cold Triton X-100-insoluble complexes perhaps equivalent to caveolae, and that GPIs may associate with members of the *src* family of cytoplasmic tyrosine kinases in these complexes (reviewed by Anderson, 1993).

Despite the promise shown by these approaches, the central questions still remain: What is the functional difference between anchoring by a GPI or by a conventional transmembrane peptide, and how does the GPI contribute to the physiological roles of the proteins that bear it? To address these questions with a protein whose function is well characterized and can be studied *in vivo*, we have focused on *Drosophila* acetylcholinesterase (AChE), an enzyme that hydrolyzes acetylcholine to terminate synaptic transmission at cholinergic synapses. In *Drosophila* there is a single form of AChE, a disulfide-linked dimer of GPI-anchored catalytic subunits, encoded by the *Ace* locus at 87E2-4 on the third chromosome. Acetylcholine is the major excitatory transmitter in the insect central nervous system, and AChE thus is widely expressed in the *Drosophila* CNS but largely excluded from the periphery. This AChE is extremely well-characterized in terms of both classical and molecular genetics and in its biochemical properties (reviewed by Restifo and White, 1990). The goal of our study is to characterize *in vivo* any functional consequences that result from the anchoring of *Drosophila* AChE by a stable transmembrane peptide domain rather than by the GPI. To approach this goal we constructed two classes of

AChE distinct from the wild type GPI-anchored form, one involving secreted, non-anchored forms, and the second anchored by the transmembrane domain of herpes simplex virus type 1 glycoprotein C (HSV-1 gC). Although gC is expressed basolaterally in HSV-infected epithelial cells, the transmembrane and small 11-residue cytoplasmic domains have no known localization signals and are not necessary for incorporation into HSV virions. We outline here the construction and biochemical characterization of these two mutant classes of *Drosophila* AChE and the results of their genetic replacement in the CNS of the fly.

We first employed recombinant DNA methods to construct two secreted forms of *Drosophila* AChE (SEC1 and SEC2) and a chimeric form anchored by the transmembrane and cytoplasmic domains of HSV-1 gC (TM-AChE). To confirm that the biochemical properties of these AChE forms were unchanged from GPI-AChE except as predicted, we made stably-transfected *Drosophila* Schneider Line 2 (S2) cells expressing each of the four forms. TM-AChE, SEC1 and SEC2 had the same catalytic activity and quaternary structure as GPI-AChE. TM-AChE was expressed as an amphiphilic membrane-bound protein resistant to an enzyme that cleaves GPI-AChE (phosphatidylinositol-specific phospholipase C), and the same percentage of TM-AChE and GPI-AChE was on the cell surface according to immunofluorescence and pharmacological data. SEC1 and SEC2 were constructed by truncating the C-terminal signal peptide initially present in GPI-AChE: in SEC1 the last 25 amino acids of this 34-residue peptide were deleted, while in SEC2 the last 29 were deleted. Both SEC1 and SEC2 were efficiently secreted and very stable in culture medium; with one cloned SEC1 line, AChE accumulated to as high as 100 mg/L. Surprisingly, 5-10% of SEC1 was attached to a GPI anchor, but SEC2 showed no GPI anchoring.

To replace GPI-AChE *in vivo* with the transmembrane or secreted forms, we returned their coding sequences to the *Drosophila* germline by transformation with P element transposon vectors. A collection of null and severely hypomorphic lethal alleles of the *Ace* locus were used to provide a genetic background that completely lacked or had greatly reduced levels (>95%), respectively, of GPI-anchored AChE. Genetic rescue crosses were performed with flies carrying *Ace* lethal mutations on the third chromosome and transgenes encoding the wild type or mutant forms on the first and/or second chromosomes. In each case the transgene was a minigene construct consisting of the wild type or chimeric *Ace* cDNAs driven by 1.5 kb of 5' genomic DNA from the *Ace* locus, a genomic segment sufficient to direct appropriate nervous system expression (Hoffman et al, 1992). A summary of these rescue crosses is provided in Table I. *Ace* null alleles die during late embryogenesis, but the GPI-AChE minigene could rescue these null alleles with the transgene typically expressing 12-40% of the normal AChE activity in wild-type flies. There was a linear gene dosage effect with the GPI-AChE minigene where two copies produced twice the activity of one. Flies expressing 20% or less AChE activity appeared normal in viability and behavior, but some individuals were bang-sensitive, that is, they underwent mechanical shock-induced paralysis. In contrast, flies expressing 20% of the normal AChE activity from a semi-lethal hypomorphic allele (*lm35*) showed no bang-sensitivity. We conclude that this phenotype is a result of some altered aspect of CNS expression from the minigene. GPI-AChE transgene inserts expressing 9% of the normal activity did not rescue the null alleles. However, such animals did not die as embryos but completed development through metamorphosis and failed to eclose, defining a threshold level of AChE for adult viability. Bang-sensitivity, a specific phenotype shown by other *Drosophila* neurological mutants, has never been reported for *Ace* mutants or other genes involved

with cholinergic transmission. Because some extent of bang-sensitivity was observed for all inserts and all classes of minigene construct (see below), it is highly unlikely that the phenotype is a result of insertion of the transgenes into known bang-sensitive loci, of which there are at least four (*bang-sensitive*, *bang-senseless*, *easily shocked*, and the gene for (Na⁺, K⁺)ATPase α subunit).

SEC1 AChE transgenes were unable to rescue null alleles even with 6 copies of transgene, which provided only 9-10% of the normal AChE activity. Unlike animals with an equivalent level of activity from the GPI-AChE transgene, these SEC1-expressing animals died as larvae and were rarely observed as pupae. Combinations of severely hypomorphic alleles expressing GPI-AChE at <5% of the normal activity were partially rescued by SEC1, however, producing adults that had 12-15% of the normal AChE levels. These SEC1-rescued flies were abnormal in behavior with coordination defects, showed a higher frequency of bang-sensitivity, and often died several days after eclosion. Interestingly, there was not a linear increase in AChE activity with increasing SEC1 transgene dosage. Additionally, analysis of AChE in brain extracts from these flies by non-denaturing polyacrylamide gel electrophoresis showed a disproportionately low level of hydrophilic AChE. These data suggest that although the SEC1 AChE is very stable when secreted in cell culture, it is either degraded by neurons or is unstable in the extracellular matrix of neuropil.

TM-AChE transgenes providing 15-20% of the normal AChE activity could only partially rescue null alleles, a clear phenotypic difference from transgenic flies expressing a comparable level of GPI-AChE. These TM-AChE flies either failed to eclose or died shortly after eclosion. Furthermore, eclosed adults were highly bang-sensitive and sometimes would undergo spontaneous paralysis. In contrast, when AChE activity levels of >30% of normal were obtained with four copies of TM-AChE transgene, rescue efficiencies were essentially identical to that for wild type minigene. However, these animals still displayed subtle coordination defects that can best be described as a tendency to become stuck in their food; they were also bang-sensitive, but no more so than GPI-AChE-rescued flies. Light-level immunocytochemistry of adult brain expressing TM-AChE or GPI-AChE appeared identical. Immunoelectron microscopic localization of both GPI-AChE and TM-AChE also appeared very similar: Both enzymes were found to be on the extracellular face of neuronal plasma membranes. Synaptic and extrasynaptic immunoreactivity was observed for both forms, and no labeling of intracellular structures was observed for either form. Apparently, TM-AChE was correctly sorted by fly neurons and not targeted to any aberrant membrane domains. We have thus far found no cellular basis for the phenotypes observed in TM-AChE-expressing flies.

The fact that TM-AChE was apparently sorted identically to the GPI-anchored form *in vivo* demonstrates that in this system the GPI anchor has a primary function unrelated to protein sorting. However, the GPI anchor must be necessary for some subtler aspect of AChE physiology because its replacement in flies expressing TM-AChE results in abnormal behavior. Unfortunately due to the complexity of insect neuropil and the small size of the *Drosophila* brain, it may be very difficult to define this subtle cellular function. While this is one of the weaknesses of a genetic approach, our data suggest that some of the other hypothetical functions of GPIs should be addressed with the vigor that the sorting function has received.

genotype	enzyme activity	viability	behavior
<i>lm35/Df(3R)l26d</i>	20%	6%	normal
wt transgene A or B; null/Df	18-40%	20-50%	normal/occasional MSIP*
wt transgene C; null/Df	9%	0	delayed pupariation; complete metamorphosis but don't eclose
wt transgene C; hypo <i>i</i> /hypo <i>j</i>	12%	29%	normal/ occasional MSIP
SEC transgene; null/Df	9-10%	0	occasional adult ecloses but drowns in food
SEC transgene; hypo <i>i</i> /hypo <i>j</i>	15%	39%	normal; frequent MSIP; early death
TM transgene; null/Df	18%	7%	if eclose, live <5 days; MSIP with slightest insult
TM transgene; hypo <i>i</i> /hypo <i>j</i>	22%	20%	normal; frequent MSIP
TM 4 copies; null/Df	31%	34%	normal; occasional MSIP; early death

Table I: Summary of genetic rescue crosses with GPI-AChE, SEC1, and TM-AChE minigenes. The results presented are averages and ranges of data from many crosses with a variety of transgene inserts for each of the three classes in combination with a panel of null and severely hypomorphic alleles. To provide a background completely lacking GPI-AChE, null alleles were combined with *Df(3R)l26d* (null/Df), a deletion which removes the *Ace* locus along with 6 other genes. Backgrounds providing 5% of normal GPI-AChE activity or less were obtained with several heteroallelic combinations of the severe hypomorphs (hypo *i*/hypo *j*). * MSIP, mechanical shock-induced paralysis or bang-sensitivity.

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**ABSTRACTS
POSTER PRESENTATIONS**

All posters will be displayed from Tuesday through Thursday. The poster presenters should be at their posters according to the following schedule:

- Sessions 1-2: Tuesday from 10:00 to 10:30*
- Sessions 3-4: Tuesday from 15:00 to 15:30*
- Sessions 5-6: Wednesday from 10:30 to 11:30*
- Session 7: Wednesday from 15:30 to 17:30*
- Session 8: Thursday from 10:20 to 10:50*

**POSTER SESSION 1:
(P1.1-P1.20)**

RECEPTOR ASSEMBLY AND STRUCTURE

P1.1 ACETYLCHOLINE RECEPTOR-AGGREGATING ACTIVITY OF AGRIN ISOFORMS AND LOCALIZATION OF ACTIVE SITE. Gesemann, M., Denzer, A.J., and Ruegg, M.A. Dept. Pharmacology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland.

Agrin is a basal lamina protein that induces formation of postsynaptic structures at the neuromuscular junction. Alternative splicing of chick agrin mRNA at two sites, A and B, generates five proteins that are differentially distributed among pre- and postsynaptic cells. Nonneuronal cells express isoforms which lack amino acids at the B-site, while motor neurons express high levels of isoforms with inserts at sites A and B. We have compared the synaptogenic activity of these agrin isoforms using acetylcholine receptor (AChR) aggregation assays. On chick myotubes, we find that the most active C-terminal half of agrin contains 4 amino acids at the A-site and 8 amino acids at the B-site (agrin_{A4B8}). Agrin_{A4B8} induces half maximal AChR-clustering at concentrations of ~ 25 pM. Agrin_{A4B19} is 4-fold less active and agrin_{A4B11} is 160-fold less active. Little or no activity was detected for agrin_{A4B0} and agrin_{A0B0}. Mature chick agrin_{A4B8} and recombinant agrin_{A4B8} of the electric ray had the same specific activity as the C-terminal half of chick agrin_{A4B8}. When tested on the mouse myogenic cell line C2C12, specific activities of recombinant agrin isoforms were similar to those observed on chick myotubes. Furthermore, N-terminal deletion constructs revealed that the C-terminal 45 kD fragment of agrin_{A4B8} was sufficient to induce AChR-clustering at similarly low concentrations and that a 21 kD C-terminal fragment was still active but required approximately 400 x higher concentrations. These experiments show that 1) agrin isoforms synthesized by motor neurons are highly active in AChR aggregation, while isoforms synthesized in nonneuronal cells show little or no activity, 2) this activity is independent of the N-terminal half of agrin and the species examined and 3) the B-site in conjunction with a region homologous to the G-domain of the laminin α 1 chain is sufficient for this activity. This work was supported by Swiss National Science Foundation #31-33697.92

P1.2 AGRIN-INDUCED AChR CLUSTERING IN MOUSE C2 MUSCLE CELLS REQUIRES TYROSINE PHOSPHORYLATION Ferns, M., Deiner, M., & Hall, Z. Dept. Physiol., UCSF, SF, CA 94143-0444

Agrin is proposed to be the nerve-derived factor that initiates acetylcholine receptor (AChR) clustering at the developing neuromuscular junction. Although experiments in chick muscle suggest that agrin-induced AChR clustering involves tyrosine phosphorylation, evidence for a similar process in mammalian muscle has been lacking. We have therefore tested the effect of tyrosine kinase inhibitors on agrin-induced AChR clustering in C2 mouse myotubes.

AChR clusters were induced in cultured C2 myotubes using a soluble form of neural agrin (C-Ag_{12,4,8}), and were visualized with rhodamine-conjugated alpha bungarotoxin. Agrin-induced clustering was blocked in a dose-dependent manner by two tyrosine kinase inhibitors, herbimycin A and staurosporine. Several other tyrosine kinase inhibitors that were tested had no effect on clustering. Treatment with herbimycin and staurosporine at concentrations of 0.5 μ M and 10 nM, respectively, completely inhibited the 9-11 fold increase in AChR clustering caused by agrin. Moreover, herbimycin and staurosporine also caused an almost complete dispersal of pre-existing clusters that had been induced by 1-2 day agrin treatment. In all experiments, the remaining AChR and 43kD protein clusters were always colocalized, indicating that the inhibitors acted to prevent or disperse the clustering of both proteins in concert. These results indicate that tyrosine phosphorylation is necessary for the formation and maintenance of agrin-induced AChR clusters in mammalian muscle, and suggests an important role for tyrosine kinases in agrin signaling in early neuromuscular junction formation. We are currently investigating what proteins are tyrosine phosphorylated in response to agrin, and whether their phosphorylation is a required step for clustering.

P1.3 DYSTROGLYCAN BINDS NEURAL AND MUSCLE ISOFORMS OF AGRIN. Sugiyama, J.E., Bowen, D.C., and Hall Z.W. Program in Neuroscience, Dept. of Physiology, University of California, San Francisco, CA 94143-0444.

The formation of acetylcholine receptor (AChR) clusters on the muscle surface underlying the nerve terminal is one of the first signs of differentiation at the neuromuscular junction. Agrin, which is secreted by neurons and incorporated into the synaptic basal lamina, induces clustering of AChRs and other synaptic proteins. There are multiple isoforms of agrin which have different AChR clustering activities; only neurons synthesize the most active form (agrin_{4,8}), while the major form found in muscle (agrin_{0,0}) is less active. Here, we demonstrate the interaction of both nerve and muscle forms of agrin with a muscle surface protein, alpha-dystroglycan.

To biochemically characterize agrin-binding proteins from muscle membranes, *Torpedo* and C2 myotube membrane proteins were separated by SDS-polyacrylamide gels, transferred to nitrocellulose and probed with labeled agrin or unlabeled agrin followed by an agrin Western blot. Both muscle and neural agrin bound to a single component in these membranes which was identified as alpha-dystroglycan. Agrin bound to purified alpha-dystroglycan and antibodies to alpha-dystroglycan blocked both muscle and neural agrin binding. However, these antibodies could not block agrin-induced AChR clustering on muscle cells. Furthermore, the binding was specific, saturable and calcium dependent; characteristics similar to those required for agrin-induced AChR clustering. Both muscle and neural agrin isoforms bound to alpha-dystroglycan with similar affinities (in the nanomolar range), in contrast to the differential concentrations required for AChR clustering activity. Thus, although the major agrin-binding protein in *Torpedo* and myotube membranes is alpha-dystroglycan, the physiological function of this interaction is unknown. We are now in the process of determining the role of muscle agrin and alpha-dystroglycan in AChR clustering.

P1.4 THE SYNAPSE-ORGANIZING MOLECULE AGRIN IN THE DEVELOPING AVIAN RETINA. Kröger S., Horton S.E.# and Honig, L.S.#. +Max-Planck Institute for Brain Research, Deutschordenstrasse 46, 60528 Frankfurt, Germany; and #Dept. of Neurobiology, Stanford University Medical School, Stanford, CA 94305-5401.

Agrin induces the formation of postsynaptic specializations including aggregates of the AChR at the developing neuromuscular junction (NMJ). Alternative splicing of agrin mRNA at positions "A" and "B" yields several agrin isoforms which differ in their distribution and in their AChR aggregating activity. Because of its presence in the central nervous system (CNS), it has been hypothesized that agrin might also induce postsynaptic specializations at neuron-neuron synapses. Using the retina, we have examined the role of agrin in the CNS. Injection of polyclonal anti-agrin Fab fragments into the vitreous humor of embryonic (E3 to E20) chick eyes specifically labels extracellular agrin. Staining is seen in the optic fiber layer (which contains retinal ganglion cell [RGC] axons), and in inner (IPL) and outer (OPL) plexiform layers, as they form, during the periods of synaptogenesis. The inner limiting membrane (the basal lamina separating the vitreous humor from the neural retina) is also stained at all developmental stages. Nuclear retinal layers (which contain few if any synapses) remain unstained. Since RGC axons showed agrin immunoreactivity on their surface *in vivo* and in culture, RGCs were co-cultured with chick myotubes: this did not result in induction of AChR aggregates. But the developing retina does contain, by PCR analysis, mRNAs for active agrin isoforms. The predominant mRNA type however was the A4B0 isoform which is inactive in AChR aggregation. *In situ* hybridization shows these agrin mRNAs are present in the RGCs and inner nuclear layers, which correlates well with the optic fiber and IPL staining for protein. The pattern of agrin expression in the developing retina is consistent with the hypothesis that agrin isoforms might have a role in synapse formation in the CNS similar to that at the NMJ.

P1.5

MULTIPLE SIGNAL PATHWAYS GENERATED BY ASSEMBLY OF TERMINAL COMPLEMENT COMPLEXES (TCC) IN THE PLASMA MEMBRANE
Florin Niculescu, Horea Rus, and Moon Shin, University of Maryland, School of Medicine,
Department of Pathology, Baltimore, MD 21201

Assembly of sublytic TCC induce hydrolysis of major membrane phospholipids with the release of prostanoids and generation of signal messenger molecules like DAG and ceramide. TCC assembly induced dose- and time-dependent increase of GTP γ S binding and GTP hydrolysis in purified plasma membranes of JY cells. Heterotrimeric G protein activation was noted first with C5b-7 assembly and increased further with C5b-8 and C5b-9 formation. PTX but not CTX induced ADP-ribosylation of a 41 kDa G α subunit which was reduced to 20% in the presence of inserted TCC. Immunoprecipitation of TCC-carrying cell lysates with antibodies to C5b-9 neoantigen, to C7 or C8 revealed the presence of PTX sensitive ADP-ribosylated G α /G α o. Ras activation was also found associated to sublytic TCC tested by GTP/GDP ratio in immunoprecipitates with anti-ras and anti-TCC antibodies. Raf-1 activity tested on H1 and MAPK (ERK1) tested on MBP phosphorylation were found increased in the first 15 min after TCC assembly in the plasma membrane. The biological significance of these multiple signal pathways generated by TCC was tested by DNA-synthesis, significantly increased, activation of cyclin-dependent kinases (cdc2, cdk) as well as a significant activation of endocytosis and exocytosis of membrane vesicles containing inserted TCC complexes. Assembly of TCC in the plasma membrane of nucleated cells is followed by a complex signaling pathway prone to escape the cell from complement induced cell lysis.

P1.6

ORAL TOLERANCE IN EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS (EAMG). Provenzano, C.†^o, Wells, G.†*, Peng, X.†, Lindstrom, J.†
†Department of Neurosciences and * Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, USA, and ^oInstitute of General Pathology, Catholic University, Rome, Italy.

The goal of treatment in any autoimmune disease is to specifically block the pathological response without affecting the normal one. Present pharmacological treatments are far away from this goal. The induction of tolerance by means of oral administration of the antigen is a new therapeutic approach which may combine substantial specificity with the absence of side effects. It has already been tested in experimental models of human autoimmune diseases such as experimental allergic encephalomyelitis, adjuvant arthritis, experimental allergic uveoretinitis and diabetes in NOD mice, as well as in two phase-one trials in humans with multiple sclerosis and rheumatoid arthritis. Moreover, it has been demonstrated that oral tolerance to the *Torpedo* AChR can effectively prevent the induction of EAMG in Lewis rats. Results from this model, however, cannot be directly applied to humans, as it is well known that the two antigens are only partially cross-reactive. The large doses that would be required to treat the human disease and the relative scarcity of natural sources of AChR prompted us to test the possibility of using recombinant fragments of the human AChR to induce oral tolerance to EAMG. Experiments are in progress to determine which fragments and dosages can induce tolerance without worsening the myasthenic symptoms.

The financial support of the Myasthenia Gravis Foundation and Telethon Italy (#532 to C.P.) and NIH and MDA (to J.L.) is gratefully acknowledged.

P1.7 EFFECT OF MICROINJECTION OF ANTIBODIES TO THE RECEPTOR-ASSOCIATED 43K PROTEIN ON THE STABILITY OF AChR CLUSTERS OF RAT MYOTUBES.
Bezakova, G., Bloch, R.J., Dept. of Physiol., U. MD Med. Sch., Baltimore, MD 21201

Nicotinic acetylcholine receptors (AChRs) are localized at high concentration in the postsynaptic membrane of the neuromuscular junction (NMJ). The receptor-associated 43K protein (43K) has been proposed to anchor receptors at postsynaptic sites, but it has not yet been shown to be necessary for AChR clustering. To address this question, we microinjected anti-43K antibodies into cultured rat myotubes, which form large, substrate-associated clusters of AChR that resemble the aggregates of AChR present at the embryonic NMJ. We tested mouse monoclonal antibodies against the C-terminal region of 43K (mAb 1579) and against a more N-terminal epitope (mAb 1234). Control myotubes were injected with either MOPC 21 IgG, which has no hapten specificity, or with mAb 88B, against the cytoplasmic domain of the AChR. The myotubes injected with mAbs were subsequently injected with goat anti-mouse IgG (GAM). Effects of microinjection on AChR clusters were determined by sequential observation of the AChRs, labeled with tetramethyl-rhodamine- α -bungarotoxin. Images of injected cells were recorded with a charge-coupled device (CCD) camera under reduced epifluorescence illumination. We observed considerable destabilization of AChR clusters after injection of myotubes with mAb 1579 followed by GAM. These effects varied both with the concentration of mAb injected and the time of incubation following injection of GAM. No disruption was observed unless GAM was used. In a few experiments, the myotubes injected with 1579 and GAM were stained with fluoresceinated anti-goat antibodies to localize the injected antibodies. Confocal laser scanning microscopy revealed that the injected antibodies are concentrated at AChR clusters. Thus, the injected antibodies concentrate where they would be expected to have the most profound effect on cluster morphology and stability. By contrast, injection of mAb 1234, mAb 88B, and MOPC 21 had no effects on cluster stability, even after injection of high concentrations of mAb and long periods of incubation. The effects of microinjected mAb 1579 are therefore specific. We conclude that 43K -- and in particular its C-terminal region -- is necessary for AChR clustering in muscle cells in vitro. (Supported by grants from the NIH and the Muscular Dystrophy Association.)

P1.8 THE MEMBRANE SKELETON AT AChR DOMAINS OF RAT MYOTUBES IS BASED ON BETA-SPECTRIN, IS QUANTITATIVELY SIMILAR TO THAT OF ERYTHROCYTES, AND IS LINKED TO INTRAMEMBRANE PARTICLES.
Pumplin, D.W., Strong, J.C., and Strakna, N.A. Dept. of Anatomy, Univ. of Maryland at Baltimore, Baltimore, Md., USA

We used immunogold labeling and quick-freeze, deep-etch, rotary replication to characterize the membrane skeleton at regions with high concentrations of acetylcholine receptors (AChR domains) in receptor clusters of cultured rat muscle cells. The membrane skeleton consists of a network of filaments closely applied to the cytoplasmic membrane surface. These filaments are immunogold labeled by a monoclonal antibody, VIIIF7, that recognizes a distinct isoform of β -spectrin. The filaments are 32 ± 11 nm in length and 9.5 ± 2.5 nm in diameter. Three to four filaments (mean 3.1) join at each intersection to form the network. These values are nearly identical to those reported previously for the membrane skeleton of erythrocytes (Ursitti, J.A., et al., Cell Motil. Cytoskeleton 19:227-243 [1991]). Filaments are decorated with gold particles close to one end and adjacent filament intersections could be labeled simultaneously by the mAb. The pattern of labeling observed is consistent with a computer model assuming random labeling of intersections between antiparallel homodimers of β -spectrin. Composite replicas giving simultaneous views of IMP and the membrane skeleton show that IMP lie under filaments and become aggregated when the membrane skeleton is condensed by exposure to high salt, further evidence that IMP are linked to the skeleton. Immunogold particles marking syntrophin, a 58Kd AChR-associated protein that may participate in this linkage, appear at intersections of membrane-skeleton filaments. The results suggest that filaments of the membrane skeleton are formed from antiparallel homodimers of β -spectrin, and that these filaments are linked to AChR near their ends. Supported by NIH and MDA grants to DWP.

P1.9 ROTARY REPLICATION OF FREEZE-FRACTURED SARCOLEMMMA RESULTS IN A DISTINCTIVE AChR-CORRELATED INTRAMEMBRANE PARTICLE. Paul W. Luther, Department of Physiology, University of Maryland School of Medicine, Baltimore, MD

Unidirectional platinum shadowing of freeze-fractured muscle cells produces large angular intramembrane particles (IMPs) that have been correlated with acetylcholine receptors (AChR). Although clustered AChR can be distinguished from other IMPs by their size, variability precludes the identification of individual AChR with certainty in conventional freeze-fracture preparations. Here we show that low-angle rotary replication of freeze-fractured AChR produces a distinctive IMP that is easily identified. Cultured *Xenopus* muscle cells were labeled with fluorescent α -bungarotoxin to identify AChR clusters. The cultures were fixed, cryoprotected with glycerol/polyvinyl alcohol, frozen against gold stubs in propane/isobutane at -196°C , and freeze-fractured with a double-replica device. The fractured specimens were rotated at 100 rpm while 2.5 nm of platinum was applied from an angle of 20° . Aneurial AChR clusters in these replicas had IMPs distributed into irregular dense aggregates separated by narrow zones of particle-free sarcolemma. Like the aggregates of large IMPs seen in conventional freeze-fractured *Xenopus* muscle cells, these particles correlated with α -bungarotoxin labeling. At high magnifications, the particles were dense, smooth accumulations of platinum 10-15 nm in diameter. Each particle contained a central electron-translucent area 2-4 nm across. The particles were round or pentagonal in shape. Similar IMPs were seen in clusters at the postsynaptic sarcolemma and individually in the extrasynaptic sarcolemma, where they presumably represent diffuse AChR. Other IMPs in the sarcolemma were less dense, were irregular in shape due to the granularity of the platinum coat, and did not have a central electron translucent area. The pentagonal shape and translucent area may represent the five subunits of AChR and the receptor ion channel respectively, although platinum decoration artifacts may be responsible for these features. Production of distinctive IMPs representing AChR should prove useful for studies of potential associations between AChR and peripheral membrane proteins, computational analysis of AChR distributions at the nanometer scale, and examination of the subunit organization of AChR. Supported by NSF IBN 93095 10.

P1.10 THE ROLE OF SUBUNIT DOMAINS IN ASSEMBLY AND STABILIZATION OF THE HETERODIMER FORMED BY THE ACETYLCHOLINE RECEPTOR α AND δ SUBUNITS. Hardy, S.F. and Hall, Z.W. Department of Physiology, University of California, San Francisco, CA, 94143-0444, USA.

We are studying assembly of acetylcholine receptor subunit fragments transiently expressed in COS cells. When full length α and δ subunits are expressed in the same cell, they form a ligand-binding heterodimer, with a large increase in the stability of the α subunit. Using deletions of both subunits, we are trying to determine if there are domains within the proteins that control stability, specificity of assembly and ligand binding. When an α subunit fragment containing only the N-terminal extracellular region was expressed α -bungarotoxin binding activity appeared both in cell extracts and in the culture media. However, no heterodimer was detected when the α N-terminal fragment was transfected with full length δ subunit. In contrast, an α subunit containing the N-terminal domain and the first transmembrane segment, efficiently formed a ligand-binding heterodimer when co-expressed with δ . These results suggest either that membrane attachment is necessary for the luminal N-terminal domain of the α subunit to associate with the δ subunit, or that the M1 segment of α plays a role in heterodimer formation. When a fragment of the δ subunit truncated after the first membrane domain was tested, it formed a heterodimer with full length α , but the amount was reduced. Further experiments shows that the reduced amount of heterodimer was due to a failure of the δ subunit to stabilize α , and that this stabilizing effect was due to the C-terminal tail of δ .

P1.11 **DEGRADATION RATES OF ACHRS CONTAINING GAMMA OR EPSILON SUBUNITS.** Q'Malley, J.P. and Salpeter, M.M. Section of Neurobiology & Behavior, Cornell University, Ithaca NY, 14853.

Acetylcholine receptors (AChRs) can be classified as two distinct physiological entities based on their metabolic degradation rates. The two forms have been called the Rr and Rs to designate rapidly degrading and slowly degrading AChRs respectively. The Rr degrades with a $t_{1/2}$ of 1 d while the Rs degrades with a $t_{1/2}$ of either 10 d (innervated muscle) or 3 days (denervated or cultured muscle). In rat muscle cell cultures both Rr and Rs receptors are present, with the Rs constituting ~ 10-20% of the receptor population and having a $t_{1/2}$ of 3 days. These muscle cells thus provide a good model system for studying AChR degradation. Although the temporal and spatial expression of the Rr and Rs is very similar to that of the fetal ($\alpha 2\beta\gamma\delta$) and adult ($\alpha 2\beta\epsilon\delta$) isoforms of the AChR, it has not been directly demonstrated that the γ and ϵ subunits are integral components of the Rr and Rs AChRs respectively. To determine if the Rr is the γ containing and Rs the ϵ containing AChR, we must answer two questions 1) are AChRs containing γ and ϵ subunits expressed in cultured muscle cells and 2) are their degradation rates the same as the Rr and Rs respectively?

We have purified AChRs from rat muscle cell cultures which were labeled with α -bungarotoxin before extraction with Triton X-100. The extracted receptors were affinity purified with an antibody directed against α -bungarotoxin. We then performed Western analysis on both the purified receptors and ϵ and γ subunit expressed by 293 cells (a gift from B. Randall) with antibodies directed against either the γ or ϵ AChR subunits.

We have been able to detect γ and ϵ subunits (based on identical migration with artificially expressed subunit protein) in AChR extracts from rat primaries. Thus we have preliminary evidence for the expression of both fetal and adult-type AChRs in rat muscle cell cultures, a necessary condition if subunit composition determines AChR degradation rates.

The affinity purification step during AChR extraction allows us to look at only those AChRs which are labeled with α -BGT. This will allow us to extract AChRs at various time intervals after a single labeling and follow their degradation. By measuring the loss of γ and ϵ subunit protein in this degrading population of labeled AChRs with Western analysis, we will be able to determine the degradation rates of γ and ϵ containing AChRs and their relationship to AChR metabolic stability.

In summary we have developed a bungarotoxin-based AChR purification system which has allowed us to analyze the subunit composition of surface AChRs. We will use this system to determine the contribution of the γ and ϵ subunits to AChR degradation. (NIH #NS09315.)

P1.12 **FUNCTIONAL CONTRIBUTION OF THE $\alpha 5$ SUBUNIT TO NEURONAL NICOTINIC CHANNELS: HETEROLOGOUS COEXPRESSION WITH OTHER α/β SUBUNITS AND ANTISENSE DELETION FROM NATIVE nAChRs**

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The $\alpha 5$ subunit of the chick neuronal nicotinic acetylcholine receptor (nAChR) family is broadly expressed in both the CNS and PNS (Ballivet, Berg and their colleagues) and yet was classified as "non-functional" based on heterologous co-expression studies with other β -type subunits. To test whether $\alpha 5$ could be coassembled and inserted as part of a channel forming nAChR complex, we examined the properties of nAChRs expressed in *Xenopus* oocytes injected with $\alpha 5$ RNA alone or in combination with other α and β subunits. Injection of $\alpha 5$ alone or $\alpha 5$ in combination with either $\alpha 4$ or $\beta 2$, produced no detectable current and injection of $\alpha 4$ plus $\beta 2$ RNA produced a single class of high affinity (apparent EC_{50} for ACh is 0.7 μM) channels with a conductance of 22 pS. In contrast, the co-injection of $\alpha 5$ with $\alpha 4$ and $\beta 2$ produced a new channel with subconductances of 45 and 50 pS. Analysis of the agonist concentration dependence of nAChR activation revealed that the coexpression of $\alpha 5\alpha 4\beta 2$ results in a 100 fold shift in the apparent K_m for ACh in a subset of nAChRs proportional to the $\alpha 5:\alpha 4$ ratio. Six cysteine replacements were made along TM2 of $\alpha 5$, four of which showed significant inhibition to applications of the positively charged sulfhydryl reagent MTSET. These data taken together support the view that $\alpha 5$ when expressed in conjunction with other α and β subunits is coassembled in nAChRs, modifying both the agonist affinity and conductance properties of the resultant nAChR channels. In view of these data, we have examined the role of $\alpha 5$ subunits in native neuronal nAChRs.

The properties of ACh-gated macroscopic and single channel currents were assayed in embryonic chick sympathetic neurons treated with control or $\alpha 5$ antisense oligonucleotides. Two different antisense oligonucleotides against different regions of the $\alpha 5$ subunit gene were used and produced identical results: First, $\alpha 5$ deletion alters the dose response curves to ACh, cytosine and nicotine with changes in the apparent affinity for agonist. Thus $\alpha 5$ -minus neurons have a higher apparent affinity for cytosine, ($EC_{50}=24 \mu M$) compared with control ($EC_{50}=75 \mu M$). These results are consistent with those revealed by heterologous expression of nAChRs $\pm\alpha 5$. The deletion of the $\alpha 5$ subunits also changes the profile of nAChR channel subtypes such that the 50pS class normally expressed, is eliminated. This observation suggests the participation of $\alpha 5$ in at least this channel class. The channels seen following the deletion of the $\alpha 5$ subunits include 14 pS, 33 pS and 63 pS subtypes, whose subunit composition remains to be determined.

P1.13 MULTIPLE NEURONAL NICOTINIC RECEPTOR ALPHA & BETA SUBUNIT mRNA EXPRESSION IN INDIVIDUAL INTRACARDIAC PARASYMPATHETIC NEURONS IS REVEALED BY SINGLE CELL RT-PCR

Poth, K., Cuevas, J., Nutter, T. J., Adams, D. J., Luetje, C. W., Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, FL, USA.

Neuronal nicotinic acetylcholine receptors (nAChRs) are composed of various combinations of subunits. The neuronal nAChR subunit gene family currently consists of 8 alpha and 3 beta subunits. *In vitro* expression studies show that each subunit combination has distinct pharmacological and biophysical properties. In parasympathetic neurons cultured from rat intracardiac ganglia, 3 distinct single channel conductances have been observed (Adams and Nutter, 1992, *J. Physiol. (Paris)* **86**: 67-76). In further studies, individual excised outside-out patches exhibited either the 30 pS and 22 pS, the 22 pS and 16pS, or all three conductance classes. Also, whole cell currents exhibited one of three different rank orders of potency for nicotinic agonists (Nutter and Adams, this session). These results suggest that individual intracardiac parasympathetic neurons may express different arrays of neuronal nAChR subunits.

To gain insight into the neuronal nAChR subunit mRNA expression patterns of individual neurons, we designed 4 degenerate PCR primer pairs (each directed toward a subset of the subunit mRNAs) and devised a restriction enzyme digestion strategy to identify each subunit from the amplified products (Lambolez et al., 1992, *Neuron* **9**: 247-258). A test of specificity, done on rat brain total RNA demonstrated that this strategy accurately identified each subunit. Use of this method to examine RNA isolated from rat adrenal chromaffin cells revealed expression of $\alpha 3$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ mRNAs. The cytoplasm of individual intracardiac parasympathetic neurons was extracted using a patch pipet and subjected to RT-PCR. The nAChR subunit mRNA expression pattern differed among individual neurons. The expression patterns ranged from simple ($\alpha 3$ and $\beta 4$, for example) to complex (for example: $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 7$ and $\beta 2$, or $\alpha 3$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$). The $\alpha 3$ subunit was nearly always expressed, while other subunits such as $\alpha 5$, $\alpha 7$ and $\beta 3$ were clearly expressed in some neurons and not in others. Differential nAChR subunit expression may thus account for the multiple single channel conductances and different pharmacological profiles observed in individual parasympathetic neurons from rat cardiac ganglia.

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P1.14 EFFECTS OF MUTATIONS TO RESIDUE α D200, AND OF MONOVALENT CATIONS, ON ACh BINDING AND GATING IN MOUSE NICOTINIC RECEPTORS. Akk, G., Sine, S.* and Auerbach, A. Dept. Biophysics, SUNY Buffalo and *Dept. Physiology and Biophysics, Mayo Foundation.

As part of a program to understand structure-function relationships in the gating of nicotinic ACh receptors we have studied the single-channel kinetics properties of wt and mutant mouse receptors transiently expressed in HEK 293 cells. The residue α D200 has been shown by others to influence the dose-response and binding properties of the receptor. Kinetic analysis of this mutant shows greatly reduced (>100-fold) rates of opening and dissociation from the first agonist binding site. There was only about a 3-fold increase in the apparent closing rate, and the association rates for ACh did not decrease substantially. These effects were observed with channels that were expressed with either γ or ϵ subunits. The effects do not arise from the fact that the mutation creates a new glycosylation site, because qualitatively similar changes were observed with the mutant α D200Q and the double mutant α D200N/T202V. Thus the residue α D200 strongly influences both the rate of channel opening and the rate of ACh dissociation from the first site.

We have also examined the effects of replacement of Na^+ with other monovalent cations on the gating reaction in receptors expressed with the α , β , δ , and ϵ subunits. Substitution with K^+ shifts the EC_{50} (determined from single channel Popen measurements) from 10 μM to 25 μM . This shift is not likely to arise from interactions of the ion with binding site(s) in the pore because it was apparent in depolarized patches where the current was in the outward direction. Replacement by Cs^+ causes an even more pronounced rightward shift (EC_{50} ~100 μM) in the dose-response profile. Preliminary results suggest that the shift arises from altered ACh binding rather than altered gating properties. Our working hypothesis is that these ions act as competitive inhibitors of ACh at the agonist binding site.

- P1.15 **EPITOPES WITHIN A HIGHLY IMMUNOGENIC REGION OF ACETYLCHOLINE RECEPTOR IDENTIFIED BY A PHAGE EPITOPE LIBRARY.** Barchan, D.¹, Balass, M.^{1,2}, Souroujon, M.C.^{1,3}, Kachalski-Kazir, E.^{1,2}, and Fuchs, S.¹. Depts. of ¹Chemical Immunology and ²Membrane Research and Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel, and ³The Open University of Israel, Tel-Aviv 61392, Israel.

Phage epitope libraries are large random collections of peptides, each of which is displayed on the surface of a bacteriophage particle. We have employed a hexapeptide phage-epitope library to identify epitopes for a monoclonal antibody (mAb 5.14), which is directed to a determinant within a highly immunogenic, cytoplasmic region of the α -subunit of acetylcholine receptor (AChR). Thus, mAb 5.14 may represent a significant subpopulation of the antibody response to AChR, that is the major autoantigen in myasthenia. We have selected two different peptide-presenting phages (SWDDIR-phage and LWILTR-phage) which interact specifically with mAb 5.14. This interaction is specifically inhibited by AChR and by synthetic peptides corresponding to the hexapeptides presented by the selected phages. Although mAb 5.14 binds to AChR in its native as well as its denatured form, the selected hexapeptides do not exist as such in the AChR molecule. However, three amino acid sequence homologues with these hexapeptides were shown to be present in the cytoplasmic region of Torpedo AChR. By extending the selected hexapeptides, at one or both ends, with amino acid residues flanking the hexapeptides in the phage, we obtained mimotopes with an up to two order of magnitude higher affinity to the antibody. These extended peptides were able to efficiently block the binding of mAb 5.14 to both peptide-presenting phages, to AChR and to anti-idiotypic antibodies specific for mAb 5.14. Such peptide mimotopes may be applied for the development of therapeutic agents that will specifically block antibodies that are involved in the autoimmune attack on AChR in myasthenia.

- P1.16 **TRANSMEMBRANE TOPOLOGY OF KAINATE RECEPTORS.** Wo, Z.G. and Oswald, R.E. Department of Pharmacology, Cornell University, Ithaca, NY 14853 USA.

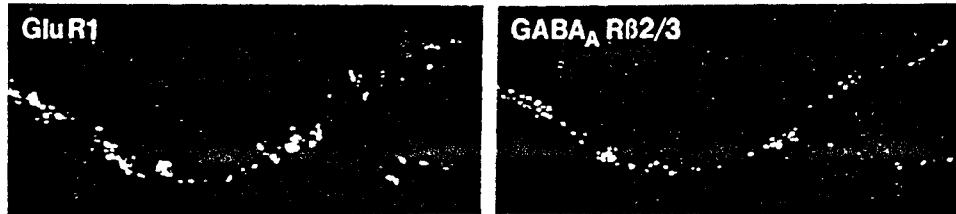
We have previously cloned cDNAs encoding two homologous kainate receptors (GFKAR α , 45 kDa, and GFKAR β , 41 kDa) from goldfish brain and proposed a topology with three transmembrane domains (Wo & Oswald, 1994, *PNAS*, in press). We have extended these studies using an *in vitro* translation/translocation system in conjunction with site-specific antibodies, site and deletion mutations. Analysis of N-glycosylation and proteolysis patterns of *in vitro* translated proteins can indicate whether a particular region is translocated at the level of the endoplasmic reticulum. The entire region between the previously proposed third and fourth transmembrane segments was found to be translocated and likely to be extracellular in mature receptors. This was based on the following results: (1) The entire segment was protected from proteinase K and trypsin digestion and could be immunoprecipitated by a site-specific antibody. (2) Functional sites for N-glycosylation are present in the C-terminal half of the segment, and (3) A mutation, constructed with an additional consensus site for N-glycosylation in the N-terminal half of the segment, was found to be glycosylated at that site. Given the fact that the N-terminal region of the protein is likely to be extracellular, this would place an even number of transmembrane segments between the N-terminus and the glycosylated extracellular segment. Results of N-glycosylation and proteolysis protection assays of GFKAR α mutations (deletion of the first three proposed transmembrane segments) indicated that the previously proposed second segment is not a true transmembrane domain. These results provide further evidence in support of a topology with three transmembrane domains.

This work was supported by a grant from the National Science Foundation (IBN-9309480). ZGW was supported on a National Institutes of Health predoctoral training grant (T32-GM08210).

P1.17

POSTSYNAPTIC CLUSTERING OF GLUTAMATE AND GABA RECEPTORS IN CULTURED HIPPOCAMPAL NEURONS. Craig, A.M., and Banker, G. Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801 and Department of Neuroscience, University of Virginia School of Medicine, Charlottesville, VA 22908.

Several immunocytochemical and physiological studies have demonstrated a concentration of neurotransmitter receptors at postsynaptic sites on neurons, but it has not been clear whether receptor clusters are selectively localized opposite terminals that release the corresponding neurotransmitter. Using antibodies against the excitatory AMPA-selective glutamate receptor subunit GluR1 and the inhibitory GABA_A receptor β 2/3 subunits, we found that these different receptor types cluster at distinct postsynaptic sites on cultured rat hippocampal neurons. The GABA_A β 2/3 subunits clustered on cell bodies and dendritic shafts opposite GABAergic terminals, while GluR1 clustered mainly on dendritic spines and was associated with glutamatergic synapses. The metabotropic glutamate receptor mGluR1 α , which is not endogenously expressed at detectable levels in cultured pyramidal cells, was expressed in the neurons from a defective herpesvirus vector. The expressed mGluR1 α , like GluR1, was restricted to the somatodendritic domain and formed postsynaptic clusters on dendritic spines. Furthermore, mGluR1 α and GluR1 formed clusters at many of the same postsynaptic sites, suggesting that similar mechanisms may regulate their localization. Chronic blockade of evoked transmitter release did not block receptor clustering at postsynaptic sites. These results suggest that complex mechanisms involving nerve terminal-specific signals are required to generate such a postsynaptic receptor mosaic. Supported by NIH NS17112, NS09248 and NS33184.



P1.18

SYSTEMATIC IDENTIFICATION OF AMINO ACID RESIDUES LINING THE CHANNEL OF THE GABA_A RECEPTOR: IMPLICATIONS FOR SECONDARY STRUCTURE AND THE LOCATION OF THE CHARGE SELECTIVITY FILTER Akabas, Myles H., and Xu, Ming. Center for Molecular Recognition, Columbia University, New York, NY 10032.

To identify the amino acid residues that line the ion channel of the GABA_A receptor we mutated, one at a time, 17 consecutive residues (259-275) in the M2 membrane-spanning segment of the rat α ₁ subunit to cysteine. We expressed the mutant α ₁ subunit with wild-type β ₁ and γ ₂ subunits in Xenopus oocytes. We probed the susceptibility of the engineered cysteine to covalent chemical modification by small, charged, sulfhydryl-specific reagents added extracellularly. We assume that among residues in membrane-spanning segments, only those residues that line the channel would be susceptible to modification by the polar reagents and that such modification would irreversibly alter conduction. We infer that the residues exposed in the channel include: Thr261, Thr262, Leu264, Thr265, Thr268, Ile271 and Asn275. When the residues in the M2 segment are plotted on a helical wheel, the exposed residues from 263 to 275 lie on one side of an α helix. Thr262 does not lie on the same side of the helix suggesting that the structure of the helix may be distorted in the region of this residue. The channel-lining residues down to Thr261 are accessible to the cationic sulfhydryl reagent applied extracellularly. This implies that the location of the charge selectivity filter is more cytoplasmic than Thr261. In the absence of agonist, the channel-lining residues are also accessible to the charged, sulfhydryl reagents: This suggests that the gate is located at a more cytoplasmic position than Thr261. Supported by NIH NS30808, AHA Grant-in-Aid, Klingenstein Award in Neuroscience and a New York Heart Association Established Scientist Award.

P1.19

UTROPHIN AND β -DYSTROGLYCAN, BUT NOT DYSTROPHIN OR β -SPECTRIN, CO-DISTRIBUTE WITH ACETYLCHOLINE RECEPTORS THROUGHOUT DEVELOPMENT OF RAT NEUROMUSCULAR JUNCTIONS

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The precise co-distribution of utrophin with acetylcholine receptors (AChRs) at the adult mammalian neuromuscular junction (NMJ) (Bewick *et al.*, 1992) suggests that it plays a role in AChR clustering during synaptogenesis. If this were the case, utrophin would be expected to co-distribute with AChRs throughout development. We have, therefore, determined the precision of the co-distribution of utrophin with AChRs at developing rat NMJs and compared it with that of other proteins related to dystrophin which are less precisely co-distributed with AChRs in the adult.

Using highly specific monoclonal antibodies detected with fluorescent probes, utrophin and β -dystroglycan (43K dystrophin-associated glycoprotein) labelling in cryostat sections was associated with all AChR clusters (labelled with Fl- α -bungarotoxin) within 24 hr of the first appearance of the clusters (embryonic day 16/17, E16/17). Labelling for dystrophin only became concentrated at most NMJs between birth and postnatal day 10 (P10) and that for β -spectrin not until P14. In *en face* views of NMJs on teased muscle fibres from postnatal muscles, the distribution of utrophin was indistinguishable from that for AChRs at all ages studied (P0-P28). The patterns of labelling for β -dystroglycan, dystrophin and β -spectrin did not come to resemble that for AChRs at most NMJs until P10, P14 and P21 respectively.

Our studies, as well as recent work by Phillips *et al.* (1993), show that utrophin and β -dystroglycan are well placed to play a role in AChR localisation from the earliest stages of AChR cluster formation. In contrast, the distribution of dystrophin and β -spectrin only comes to resemble that for the AChRs during the later stages of NMJ maturation and are thus unlikely to play an essential role in the earliest stages of synaptogenesis.

Bewick G.S., Nicholson L.V.B., Young C., O'Donnell E. & Slater C.R. (1992). NeuroReport 3:857-860.

Phillips W.D., Noakes P.G., Roberts S.L., Campbell K.P. & Merlie J.P. (1993). J. Cell Biol. 123:729-740.

P1.20

THE ROLE OF PROTEIN PHOSPHORYLATION AND DEPHOSPHORYLATION IN AGRIN-INDUCED AChR AGGREGATION. Meier T., Perez G. M.,

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Agrin, a protein released by nerve terminals at developing neuromuscular junctions, triggers the formation of specializations on myotubes in culture that contain several components of the postsynaptic apparatus, including aggregates of acetylcholine receptors (AChRs). Agrin also induces tyrosine phosphorylation of the AChR β subunit. Several treatments known to inhibit agrin-induced AChR aggregation also inhibit agrin-induced AChR tyrosine phosphorylation, suggesting that tyrosine phosphorylation plays a role in receptor aggregation. In order to understand the biochemical mechanisms that mediate agrin-induced AChR aggregation, we are currently studying the effects of inhibitors of protein kinases and protein phosphatases on agrin-induced AChR phosphorylation, aggregation, and mobility in chick and mouse myotubes in cell culture. Our results indicate that spontaneously active protein kinases and phosphatases modulate both AChR β -subunit tyrosine phosphorylation and attachment of AChRs to the cytoskeleton, and that agrin increases the activity of such kinases (or decreases the activity of such phosphatases). Thus, formation of receptor aggregates may be the result of agrin-induced foci at which kinase activity is increased (and/or phosphatase activity is decreased), leading to accumulation of phosphorylated AChRs.

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The Cholinergic Synapse:
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UNIVERSITY OF MARYLAND
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NOTES

**POSTER SESSION 2:
(P2.1-P2.19)**

SYNAPTOGENESIS AND DEVELOPMENTAL NEUROBIOLOGY

P2.1

A MAMMALIAN CULTURE SYSTEM FOR ANALYSIS OF THE EARLY STAGES OF NEUROMUSCULAR JUNCTION FORMATION. Daniels, M.P., Uhm, C.-S., Samuelsson, S.J., and Dutton, E.K. Lab of Biochemical Genetics, NHLBI, NIH, Bethesda, MD, U.S.A.

Development of the neuromuscular junction (NMJ) in the rat has been studied extensively, but most *in vitro* analysis has used amphibian or avian cultures. In order to take advantage of information on rat NMJ formation and the molecular and immunological probes for mammalian systems, we developed a culture system in which neurons from the ventral spinal cord of fetal rats are added to rat muscle cultures that contain large, branching myotubes but few non-muscle cells (Dutton et al., Soc. Neurosci. Abstr. 19: 1294 (1993)). Neurites grow extensively along the myotube surfaces, and begin to induce accumulation of acetylcholine receptors (AChR aggregation) within 6 hr of coculture. After 36-48 hr, up to 35% of the length of neurites in contact with the lower surfaces of myotubes is associated with AChR aggregates.

The ability to induce AChR aggregates belongs predominantly to developing axons, rather than dendrites, as distinguished by immunocytochemical labeling with antibodies against synaptophysin, MAP 2 and the phosphorylated neurofilament heavy subunit, as well as by morphology. These data suggest that an essential component for the induction of postsynaptic differentiation is subject to polarized expression.

Electron microscopy of sites showing neurite-induced AChR aggregation at 2 days reveal axonal profiles within furrows in the myotube surface, with which they are in contact, but only scattered synaptic vesicles are seen. The sarcolemma lateral to the neurite contact shows characteristic specializations. In 6 day-old cocultures, bouton-like expansions of the axon are separated from the myotube by a cleft (25-100 nm) containing a basal lamina, and the sarcolemma facing the bouton is highly specialized. The boutons contain accumulations of synaptic vesicles as well as dense submembrane structures resembling those at active zones. Labeling patterns obtained with synaptophysin antibodies and with uptake of the dye FM1-43 also are consistent with the accumulation of vesicles at presynaptic boutons. Thus, the induced aggregates represent sites capable of developing relatively mature synaptic structure.

A procedure has been developed to study interactions between axon growth cones and myotubes that lead to synaptogenesis. Dissociated neurons are allowed to reaggregate in suspension (Moscona, Exp. Cell Res. 22: 455 (1961)) and the aggregates (0.1-0.4 mm diameter) are pipetted on to the myotube cultures where they adhere and extend axons. Axons and their growth cones are efficiently labeled by microinjecting a solution of the lipophilic dye Fast DiO, in a dimethylsulfoxide-oil mixture, into the neuronal aggregate. Simultaneous visualization of axon growth and AChR (labeled with TRITC- α -bungarotoxin) distribution in living cultures is achieved with low incident illumination and high resolution digital imaging.

P2.2

NEURAL CONTROL OF JUNCTIONAL ACETYLCHOLINE RECEPTORS NUMBER: ROLE OF MUSCLE ACTIVITY. Andreose, J.S.†*, Fumagalli, G.*, and Lomo, T.† Inst of Neurophysiology, University of Oslo, Norway and *CNR Ctr of Cytopharmacology, Dept of Pharmacology, University of Milano, Italy.

At the neuromuscular junction (nmj), innervation plays a role in the induction and maintenance of several properties of the acetylcholine receptor (AChR), including distribution, metabolic stability and number. The nerve terminal may affect the properties of AChRs in the postsynaptic membrane through released molecules and evoked muscle activity. To examine the relative role of these two factors in the control of junctional AChR number we compared counts of ¹²⁵I-alpha-bungarotoxin binding sites in muscles after denervation, denervation and electrical stimulation, paralysis by botulinum toxin (BoTX), or tetrodotoxin (TTX). After denervation muscles lost 50 to 65% of their junctional AChRs in 33 and 57 days, respectively. Endplates paralysed by BoTX or TTX for 33 days lost a similar number of junctional AChRs (54% and 55%). Direct high frequency muscle stimulation (100 Hz) maintained a normal number of junctional AChRs for at least 2 months when the stimulation started on the day of denervation. When the stimulation started later, the protective effect on AChR number disappeared gradually. Direct low frequency muscle stimulation (10 or 20 Hz) from the day of denervation did not maintain a normal number of junctional AChRs. Endplates which had been denervated and stimulated for 18 days lost AChRs during the next 15 days in the absence of stimulation. Endplates which had been continuously blocked by BoTX for 33 days lost AChRs during stimulation at 100 Hz from day 18 to 33. The results suggest that the number of AChRs decreases following denervation and paralysis by TTX or BoTX because these treatments remove a trophic influence from the nerve, and that such influence can be maintained only if muscles are kept active by direct high frequency stimulation from the time of denervation. We conclude that evoked muscle activity acts in concert with nerve derived signals in the control of junctional AChR number at the nmj.

P2.3 **LONG TERM MAINTENANCE OF NERVE TERMINAL FUNCTION IN THE ABSENCE OF MUSCLE FIBERS IN THE FROG.** Dunaevsky, A. and Connor E. A. Neuroscience and Behavior Program, Dept. Univ. of Massachusetts, Amherst, MA 01003.

Although some of the signals involved in forming and maintaining the synaptic specializations of a target cell are now identified, little is known about the processes that lead to the differentiation and maintenance of the presynaptic nerve terminal. Here we have investigated the role of the muscle fiber in the maintenance of nerve terminal function. We demonstrate that motor nerve terminals permanently deprived of targets maintain the ability to release and recycle synaptic vesicles in response to stimulation. We assayed nerve terminal activity in preparations of innervated muscle basal lamina sheaths using a fluorescent dye FM1-43 that stains nerve terminals in an activity-dependent fashion. Innervated basal lamina sheaths were prepared by excising cutaneous pectoris muscle fibers without damaging nerve terminals. X-irradiation prevented regeneration of the muscle fibers. One to five months after muscle fiber excision, the function of target-deprived nerve terminals was tested by exposure to FM1-43 dye in high K^+ Ringer's solution. Synaptic sites were identified with rhodamine-labeled peanut agglutinin which stains terminal Schwann cell and synaptic basal laminae. Like intact preparations, nerve terminals deprived of target for 1-5 months incorporated FM1-43 when stimulated. Further, the same terminals were destained by either nerve stimulation or depolarization with high K^+ Ringer. The ability of these nerve terminals to release and recycle synaptic vesicles indicates that the molecular machinery required for vesicular release is maintained in a functional state for long periods of time in the absence of the target muscle fibers. This work was supported by NIH NS26879.

P2.4 **LONG TERM MONITORING OF INDIVIDUALLY TRANSFECTED MUSCLE FIBERS AND THEIR SYNAPSES IN LIVING MICE.** van Mier, P., Donoghue, M.J., Sanes, J., Balice-Gordon, R., and Lichtman J.W. Dept. Anat. & Neurobiol., Washington University School of Medicine, St. Louis, USA.

To better understand the factors that regulate synapses at a molecular level, we have begun to study individual transfected muscle fibers in the sternomastoid muscle of living mice. Muscle fibers were initially transfected by pressing DNA encoding for bacterial β -galactosidase (*lacZ*) with a needle into the muscle (van Mier et al. 1993, Soc. Neurosci. Abstr. 19:1271), and found to be transfected as early as 2 days after application. In later experiments, identified muscle fibers were transfected near the neuromuscular junctions by intracellular injection. Both methods of DNA application resulted in stable transfection for well over 8 months (as long as studied). X-gal staining of transfected muscle fibers showed blue staining product several millimeters along the muscle fiber at the injection site and, unexpectedly, a single deep blue stained nucleus (probably the transfected nucleus) at the site of DNA application. Most of the muscle fibers (87%) that survived the intracellular DNA injection (44% of the injected fibers) expressed the *lacZ* gene. Injection of *lacZ* DNA into normal regenerating and dystrophic (*mdx*) muscle fibers seemed equally effective, despite the suggested fragility of muscle fibers in *mdx* mice (Petrov et al. 1993, PNAS 90:3710-3714). In *mdx* mice transfected muscle fibers were found for at least 3 weeks after injection.

To modify the phenotype of muscle fibers in the adult, we have begun to intracellularly inject fibers with DNA encoding for nerve growth factor (NGF) which was tested for expression by transfecting and immunostaining QT6 quail fibroblasts. Individual muscle fibers were injected with DNA solution containing 1 μ g/ml Fluoresceinated Dextran which remained detectable as small perinuclear accumulations in muscle fibers for up to 8 months after injection. Our first observations show that transfection with NGF DNA induced sprouting of sympathetic axons innervating blood vessels surrounding 6 of 22 successfully injected muscle fibers. This sprouting could not be induced by injecting *lacZ* DNA. We are currently trying to inject DNA encoding for IGF-2, CNTF, BDNF, NT-3 or truncated acetylcholine receptor subunits (Verrall and Hall 1992, Cell 68:23-31; all these DNAs expressed protein in transfected QT6 cells).

LacZ expressing muscle fibers are detectable in living mice (van Mier et al. 1993) and we hope to eventually use DNA constructs containing genes for a reporter and a synaptically relevant protein, which will allow us to confirm transfection in vivo and to determine the expression site.

P2.5

NEUREGULIN IS A CANDIDATE FOR THE BASAL LAMINA SIGNAL THAT ACTIVATES SYNAPSE-SPECIFIC GENE EXPRESSION. Jo, S. M.¹, Marchionni, M. A.², and Burden, S.J.^{1*}. ¹Center for Blood Research, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115 and ²Cambridge Neuroscience Inc., One Kendall Square, Building 700, Cambridge, MA 02139.

Motor neurons induce the aggregation of acetylcholine receptors (AChR) at neuromuscular synapses. We have shown that a signal in the synaptic basal lamina stimulates transcription of the AChR δ subunit gene in the synaptic nuclei of skeletal myofibers (Jo and Burden, *Development* **115**: 673-680, 1992). Because the neuregulin gene is known to encode an activity (ARIA) that stimulates AChR synthesis (Falls et al., *Cell* **72**: 801-815, 1993), we were interested in determining whether products of the neuregulin gene might be the basal lamina signal. Here, we show that neuregulin (rh GGF2, Marchionni et al., *Nature* **362**: 312-318, 1993) activates transcription of the AChR δ subunit gene in C2 myotubes stably transfected with a gene fusion between 181 bp of 5' flanking DNA from the AChR δ subunit gene and the human growth hormone (hGH) gene. In contrast, neuregulin does not increase hGH expression from C2 myotubes stably transfected with a metallothionein promoter-hGH gene fusion. Thus, neuregulin activates AChR gene expression, and the *cis*-acting elements for neuregulin-mediated AChR δ subunit gene expression are contained in the same 181 bp that confers synapse-specific expression *in vivo*. In addition, immunocytochemical studies with antibodies to neuregulins show that neuregulins are concentrated at synaptic sites, and that neuregulins remain at synaptic sites following denervation. In contrast, components of the nerve terminal, such as the synaptic vesicle protein (SV2) and neurofilaments (SMI-31), are lost from synaptic sites after denervation. These data are consistent with the idea that neuregulin is the basal lamina signal that stimulates AChR gene expression in synaptic nuclei *in vivo*.

P2.6

DEVELOPMENTAL CHANGES OF ACETYLCHOLINE RECEPTOR GENE EXPRESSION IN *TORPEDO* ELECTRIC ORGAN AND SKELETAL MUSCLE. Asher, O.* Fuchs, S.* and Souroujon, M.C.** Dept. of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel and **The Open University of Israel, Tel Aviv 61392, Israel.

The mRNA levels of acetylcholine receptor (AChR) were followed during embryonic development of *Torpedo* skeletal muscle and its homologue, the electric organ. Northern blot analysis at different stages of development demonstrated that in the electric organ there was a large increase in the mRNA levels of all AChR subunits during development. The largest change occurred concomitantly with synaptogenesis in embryo 4-6 cm long. On the other hand, in the muscle we observed only small changes in AChR transcripts during development. In order to find out whether myogenic factors are responsible for the burst in the expression of AChR during differentiation of the electric organ of *Torpedo*, we have compared the expression of MyoD and MRF4 in the electric organ and in the muscle during embryonic development. We found that the mRNA levels of MyoD and MRF4 changed only slightly during development and their amounts were quite comparable in the electric organ and muscle. These results demonstrate that in the electric organ the mRNA levels of the myogenic factors do not correlate with the expression of AChR subunits. It thus seems that these myogenic factors are not directly responsible for the large increase in the AChR mRNA levels in the electric organ during embryonic development and may implicate that another regulatory mechanism governs this unique burst in AChR expression.

P2.7

GENES THAT ARE DIFFERENTIALLY EXPRESSED DURING SYNAPTOGENESIS IN THE EMBRYONIC CHICKEN CILIARY GANGLION: ISOLATION BY RT-PCR.

Ikononov, O.C., and Jacob, M.H. Worcester Fnd. for Exp. Biol., Shrewsbury, MA, U.S.A.

The chicken ciliary ganglion (CG) is a useful system to study the separate role of innervation and target tissue interactions in regulating gene expression during neuronal differentiation and synapse formation *in situ*. Both cell-cell interactions can be prevented by microsurgical removal of the source of presynaptic inputs or the target tissues, prior to synaptogenesis. These manipulations cause substantial declines in acetylcholine receptor subunit mRNA levels relative to control neuron values. We have now developed a modified differential display technique to identify other genes in the CG whose expression during synaptogenesis is regulated by cell-cell interactions.

Total RNA (approx. 0.5 μ gs) was extracted from CGs dissected from operated or control embryos on E 8. Standard RT-PCR was used (50°C temperature of annealing and 30 cycles of amplification) with two different sets of primer combinations: oligo (dT)₁₅ was used as 3' primer combined with 5' primers of different length and sequence, or two primers with 50% GC content and similar T_m were used. Both primer combinations resulted in multiple bands that were detected after separation of PCR products using 6% sequencing gels. Bands were selected for reamplification and further analysis on the basis of: 1) differential expression in the absence as compared to the presence of cell-cell interactions, 2) dependence on the addition of reverse transcriptase and 3) necessity of both primers for generating the PCR product.

Sequencing of cloned PCR products showed that all cDNAs amplified with oligo dT and a 5' primer were surrounded by the 5' primer alone. Even when two different 5' and 3' primers with 50% GC content were used, several products turned out to be dependent solely on the 3' primer. More than 50% of these cDNAs were identical to portions of highly abundant RNAs, in particular, chicken ribosomal RNAs and mitochondrial transcripts. However, cDNAs were also obtained that were dependent on the two primers. These cDNAs showed identity or homology with transcripts of medium and low abundance (chicken homologs of Rab GDP dissociation inhibitor, gephyrin, embryonic myosin), or are unknown (75% of the products). In conclusion, this modified differential display technique allows rapid and efficient cloning of low abundance transcripts that are differentially expressed in the embryonic CG during synaptogenesis. (Supported by NIH 21725).

P2.8

SPATIAL DISTRIBUTION OF NEURONAL NICOTINIC ACETYLCHOLINE SYNAPSES (nAChS) IN GUINEA PIG PERIPHERAL AUTONOMIC GANGLIA. L.G. Ermilov, S.M. Miller, P.F. Schmalz, V.A. Lennon and J.H. Szurszewski. Depts. of Physiology and Biophysics and Immunology, Mayo Clinic, Rochester, MN, U.S.A.

Monoclonal antibody 35 (Mab35) recognizes the alpha subunit of certain nicotinic receptors in brain and peripheral ganglia (Lindstrom et al. 1987). We used Mab35 and IgG anti-immunoglobulin conjugated with Cy5 fluorophore to map the spatial distribution of nAChS on single neurons labelled intracellularly with Lucifer Yellow (LY). Confocal microscopy was used to make 30 to 70 optical sections at 1 μ m intervals through labelled neurons. LY and Cy5 images were made at each interval. 3-D reconstruction of each neuron and location of visible nAChS were made using ANALYZER image processing software. In 4 neurons of the inferior mesenteric ganglia (IMG), 4.5 \pm 1.2% (mean \pm SE) of the total membrane surface area was occupied by nAChS. Most (72%) of this receptor area was distributed along 2° and 3° dendrites. 25% of the area was on 1° dendrites and 3% on the soma. In preliminary studies of the pelvic hypogastric ganglia (PHG), nAChS occupied 6.7% of the total membrane surface area. Most (88%) of this receptor area was distributed on the soma and 12% along 1° dendrites. Intracellular Mab35 immunoreactivity (IR) was observed in a substantial number of neurons in both IMG and PHG. Thus, lumbar dorsal root ganglion (DRG) cells were studied. Cell surface Mab35-IR was not detected. However, 15% of all DRG cells had intracellular Mab35-IR. The results, together with preliminary data in which 40% of visible VIP synapses were found distributed along 1° dendrites and soma of IMG neurons, provide a 3-D map of the location of cholinergic nicotinic and peptidergic synapses. Such a differential distribution of fast nicotinic and neuromodulatory peptidergic synapses and the increase in R_m that VIP causes would account for conversion of subthreshold nicotinic F-EPSPs to postsynaptic action potentials observed when VIP synapses are transmitting. The difference in distribution and surface areas of nAChS between IMG and PHG neurons may account for the occurrence of multiple, subthreshold F-EPSPs with different latencies seen in IMG neurons during nerve stimulation, and for the occurrence of few but large amplitude F-EPSPs that nearly always initiate postsynaptic spikes in PHG neurons. Intracellular Mab35-IR may reflect intracellular trafficking of nicotinic receptors. (NIH Grants DK17632 and CA37343.)

P2.9

REGULATION OF BDNF EXPRESSION IN THE CHICK OPTIC TECTUM BY NEUROTRANSMITTERS DURING SYNAPTOGENESIS. Herzog, K.-H. and Barde Y.-A. Department of Neurobiochemistry, Max-Planck Institute for Psychiatry, 82152 Planegg-Martinsried, Germany.

Previous studies have shown that the mRNA of brain-derived neurotrophic factor (BDNF) is regulated by retinal ganglion cells (RGC) as soon as their axon terminals have reached the tectum. Since intraocular injections of tetrodotoxin influenced this regulation, we were interested to determine which neurotransmitter system contributes to the regulation of BDNF mRNA. While previous studies by others have demonstrated that kainic acid and pilocarpin up-regulate the levels of BDNF mRNA in the hippocampus of rats, we found that neither substance had any effect in the chick optic tectum at E7. In contrast, several other neurotransmitter agonists and antagonists, applied onto the chorioallantoic membrane at E6, were found to prevent the increase in BDNF mRNA normally observed between E6 and E8. While nicotinic acetylcholine receptor antagonists did not show any effect, muscarinic blockers, such as atropine or pirenzepine, reduced the BDNF mRNA to levels comparable to those seen after tetrodotoxin injections. Similar effects were obtained with muscimol, a GABAergic agonist. To exclude the possibility that muscarinic acetylcholine receptor antagonists act pre-synaptically on RGCs, pirenzepine was injected into the eye. This treatment did not lead to a decrease in BDNF mRNA levels in the tectum. Surprisingly, the ability of BDNF mRNA levels to be regulated by neurotransmitter systems was limited to the earliest stages of target encounter. After E8 for example, no changes in BDNF mRNA levels were observed in the tectum after administration of neurotransmitter antagonists, or even after optic stalk transection. These results suggest a role of BDNF during the earliest stages of synaptogenesis, as the first immature synapses between RGC axons and tectal cells are observed at E7.

P2.10

NMDA RECEPTOR ACTIVATION REGULATES THE EXPRESSION OF A NEW POU/HOMEBOX GENE, *CNS-1*. S. Shah, H. Cui, X. Lin and R.F. Balleit. Dept. of Pharmacology, Univ. of Maryland Sch. of Med., Baltimore, MD 21201.

POU/homeobox genes encode transcription regulatory proteins that are important in determining cellular phenotypes. We have identified a cDNA corresponding to a new member of the POU/homeobox gene family. RNA encoded by this new gene is predominantly expressed in the central nervous system. Thus, the protein encoded by this POU/homeobox gene may be important in specifying or maintaining the phenotype of CNS neurons. We have designated this new gene *Cns-1*. *In situ* hybridization experiments show that *Cns-1* transcripts are expressed in many different cell populations within the CNS, including cells of the cerebellum. Treatment of cells cultured from the early postnatal cerebellum with 50 μ M N-methyl-D-aspartate (NMDA) results in a 3-4 fold increase in the level of *Cns-1* RNA. Simultaneous treatment of these cells with 100 μ M amino-5-phosphonovaleric acid (APV) blocks this increase. Continued treatment of these cells with NMDA maintains this new steady state level of *Cns-1* mRNA for at least five days. Depolarization of these cells with 40 mM KCl also results in a similar increase in the level of *Cns-1* RNA. The NMDA induced increase in *Cns-1* mRNA was reduced by pretreatment with the calcium chelators, ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) or 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) tetrakis(acetoxymethyl). Transcription run-off experiments suggest that this increase in the level of RNA was due, at least in part, to an increase in transcription from the *Cns-1* gene. These studies suggest that specific activation of the NMDA receptor in cultures of differentiating CNS neurons increase *Cns-1* gene expression and that calcium entry through the NMDA channel may be required for this response. This change in *Cns-1* expression may modify phenotypic characteristics of these cultured cells. [Supported by NIH Grant NS29792]

P2.11

SYNAPTIC REMODELLING IN KYPHOSCOLIOTIC MICE (KY) Coulton, G.R.¹, Biggin, A., Skynner, M.J.¹, Gangadharan, U.², Entwistle A.³, Brown, S.D.M.¹, van Mier, P.⁴. Biochemistry¹ Charing Cross and Westminster Medical School, London, UK. Biochemistry, St. Mary's Hospital Medical School, London, UK². Ludwig Institute of Cancer Research, London, UK³. Anatomy and Neurobiology, Washington University, St. Louis, USA⁴.

Ky mice suffer from an autosomal recessively inherited (Chr. 9) chronic spinal curvature precipitated by neuromuscular disease. Weight-bearing muscles (e.g. soleus) exhibit postnatal muscle fibre necrosis and regeneration (birth to 35 days). Nerves are normal save for terminal sprouting in remodelled muscles (35 to 365+ days). This is associated with abnormally distributed acetylcholine esterase at neuromuscular junctions (nmj). Fast muscles (EDL) are relatively spared.

FITC-labelled α -bungarotoxin (α -BgTx) was used to localise acetylcholine receptor (AChR) in post-synaptic membranes of nmjs in whole muscle fibres of soleus and EDL under confocal microscopy (7-365 days). Using epifluorescence and low-light level video microscopy, vital dyes staining living nerve terminals and TRITC- α -BgTx-labelled AChR sites were visualised; dynamic events in living sternomastoid muscles were observed from six weeks onward.

AChR patches in ky were normal in both soleus and EDL until the onset of muscle necrosis in soleus (14 days) and remained normal in EDL. However, developmental "milestones" were delayed by a week in ky mice. After two weeks soleus nmjs comprised small discontinuous patches of AChR. Normal nmj structure was never re-established in ky soleus. In living ky sternomastoid muscles over a number of weeks observation, patches of AChR within nmjs shrank to be replaced by others at a distance. As necrosis is rare in ky sternomastoid, nmj instability may not simply be due to muscle fibre remodelling.

The role of protein phosphorylation in ky nmj development was assessed by immunolocalisation of phosphotyrosine residues. In normal nmjs phosphorylation was intense and constant whilst some ky nmjs (identified by α -BgTx) were undetectable with anti-phosphotyrosine. These results suggest that the ky mutation affects the phosphorylation status of nmj proteins, including AChR and is associated with nmj malformation.

P2.12

CHARACTERISATION OF EMBRYONIC STEM CELL- DERIVED NEUROGENESIS. Strübing, C., Ahnert-Hilger, G.,[#] Wobus, A.-M.,^{*} Wiedenmann, B.[#] and Hescheler, J. Institut für Pharmakologie, FU Berlin, Thielallee 69-73, D-14195 Berlin, Germany, ^{*}Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstraße 3, D-06466 Gatersleben, Germany and [#]Klinikum Steglitz, Abt. für Innere Medizin/Gastroenterologie, Hindenburgdamm 30, D-12200 Berlin, Germany.

Embryonic stem cells provide a new *in vitro* model to investigate cell differentiation and early developmental processes. Embryonic stem cells of the permanent line BLC 6 develop into cell types of all three primary germ layers, including neuronal and skeletal muscle cells, upon 4 days cultivation in 3-dimensional aggregates ("embryoid bodies"). It was shown that myogenesis in this model closely resemble the embryonic development of skeletal myocytes *in vivo* (Rohwedel *et al.*, Dev. Biol., 163 (1994) in press). Depending on the cultivation conditions (initial cell number of embryoid bodies, induction with retinoic acid) we obtained distinct differentiation pattern favouring either neuronal or mesodermal (myocyte) or both cell types.

We now characterised the BLC 6- derived neuronal cells in order to prove their potential to differentiate into mature neurons. Using the whole-cell patch clamp technique we followed the expression of ionic currents in neuronal cells from 2 - 20 days after embryoid body plating. We found that BLC 6 derived neuronal cells sequentially expressed neuron-specific voltage-dependent ion channels (Na⁺-, Ca²⁺- channels), receptor- operated channels (GABA_A-, Glycine-, NMDA-, AMPA/Kainate) and G protein- coupled membrane receptors (GABA_B-, α -adreno- and somatostatin). At the terminal stage we measured miniature and spontaneous postsynaptic currents from differentiated neurons indicating a network of synaptically coupled cells. Immunofluorescence studies revealed that terminally differentiated cells were positive for all neuronal marker proteins tested (neurofilaments, synaptophysin, synaptobrevin, N-CAM, SV2). Synaptophysin- positive processes were also detectable on neighbouring skeletal myocytes in embryoid body outgrowths. It has to be studied further if these contacts between neurons and skeletal myocytes already form functional neuromuscular synapses.

We conclude that the BLC 6 cell line is an appropriate model to study the cell lineage determination of pluripotent stem cells, neuronal and myogenic cell differentiation and allows to investigate interneuronal and neuromuscular synaptogenesis.

This work was supported by ZEBET, Bundesgesundheitsamt FRG to J.H., Grant Fo2.1-1328-122.

P2.13

COMPOSITION AND ASSEMBLY OF THE EXOCYTOTIC FUSION COMPLEX.
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A tight complex of proteins involving synaptic and plasma membrane proteins locks the synaptic vesicle in position for release at the nerve terminal (Söllner *et al* 1993). Components of the fusion complex have been shown to include syntaxin A and B, SNAP-25, synaptobrevin (VAMP), α -Snap, NSF and synaptotagmin. We now show the complex also includes Munc-18 and cellubrevin, both of which bind directly to syntaxin. SNAP-25, synaptobrevin II and syntaxin form a tight complex to which other components can bind. Thus with the many different isoforms of each of these components specificity is likely encoded for the differing vesicle interactions.

P2.14

SUBLYTIC TERMINAL COMPLEMENT COMPLEXES (TCC) ON OLIGODENDROCYTES REDUCE MYELIN PROTEIN mRNAs, INDUCE ONCOGENES AND CYCLIN-DEPENDENT KINASES ACTIVITY
Horea Rus, Florin Niculescu and Moon Shin. University of Maryland, School of Medicine, Department of Pathology, Baltimore MD 21201

Activation of TCC on oligodendrocytes (OLG) is a required process in antibody-mediated demyelination in vitro. Sublytic TCC on OLG induces LTB₄, by activating PLA₂ in Ca²⁺ and PKC dependent manner. In the present study, the TCC effect on OLG was examined for the ability of TCC to regulate the expression of myelin protein genes, oncogenes, and cyclin-dependent kinases activity. In primary cultures of rat OLG, sublytic activation of serum complement reduced accumulation of mRNA encoding proteolipid protein and myelin basic protein within 1h and caused sustained increase in c-jun, jun D, and c-fos mRNAs. These effects were attributed to TCC, as demonstrated by experiments using C7 deficient serum \pm C7. Increased oncogene expression was associated with increased BrdU and ³H-Thymidine incorporation and enhanced cdc2 kinase activity tested on H1 histone. TCC-induced cdc kinase activity was 5-fold at 4h and 3.5-fold at 8h. The immunoprecipitates with anti-cdc2 from both O-2A and OLG contained cyclin B, E and D. The induced O-2A differentiation was associated to an increase of cdc2 kinase activity in the first 18 h, followed by a decrease to the lower levels found in OLG. This increase of cdc2 kinase activity was also associated with an increase of cyclins B, D1 and E content in anti-cdc2 immunoprecipitates. Sublytic TCC on OLG induced also a sharp and transient decrease of cyclin B1 associated H1 kinase activity. The OLG progenitor cells cultured in growth medium revealed increased oncogene expression and cdc2 kinase activity without myelin protein mRNA expression. Therefore, sublytic TCC attack appeared to reverse OLG from differentiation state to the precursor-like state, which is characteristic for the O-2A progenitors and thus could contribute to the demyelination process.

P2.15

CHRONIC AND ACUTE EFFECTS OF NEUROTROPHINS ON SYNAPSE DEVELOPMENT AND FUNCTION. Wang, T., Kim, H.G., Xie, K., Olafsson, P., and Lu, B. Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ, 07110 USA.

Neurotrophins (including NGF, BDNF, NT-3, NT-4/5) have traditionally been regarded as slowly acting signals essential for neuronal survival and differentiation. However, little is known about their role in synaptic development and function. We have investigated both acute and long-term effects of neurotrophins on the physiological properties of synapses during development. Using *Xenopus* nerve-muscle co-cultures, we found that BDNF and NT-3, but not NGF, significantly potentiated spontaneous and evoked synaptic activities in neuromuscular synapses. Most of the synapses treated by the neurotrophins exhibited a bell-shaped amplitude distribution of spontaneous synaptic currents, which reflects mature quantal secretion. Impulse-evoked synaptic currents also showed higher and more consistent amplitudes, indicative of more mature and reliable synapses. Moreover, the neurotrophins enhanced the expression of synapsin I, a synaptic vesicle associated protein involved in synaptic maturation. The neurotrophin effects appear to be mediated by the Trk family of receptor tyrosine kinases, primarily through a presynaptic mechanism. These results suggest that BDNF and NT-3 promote functional maturation of synapses.

To study the acute effects of neurotrophins on the functional properties of synapses, we used cortical neurons in culture. We found that NT-3 rapidly increased the frequency of spontaneous action potentials, and synchronized synaptic activities in developing cortical neurons. In addition, the inhibitory synaptic transmission mediated by GABA_A receptors was reduced by NT-3. Thus, the excitatory effects of neurotrophins on spontaneous action potentials were attributable to a reduction of GABAergic transmission. Our finding, together with previous reports of the rapid regulation of CNS neurotrophin expression by neuronal activity, suggest a new mechanism for modulation of synaptic transmission and activity-dependent synaptic plasticity.

P2.16

ROLE OF SNAP-25 IN AXONAL ELONGATION AND MAINTENANCE AS DEMONSTRATED BY AXONAL IMMUNOREACTIVITY IN DEVELOPMENT AND IN REGENERATING AXONS OF SKIN. Oyler G.A., Hsieh S-T., Drachman D.B., Griffin JW. Department of Neurology, Johns Hopkins, Baltimore MD, 21000.

SNAP-25 is a protein originally identified as a component of the cytoplasmic surface of the presynaptic membrane in a diverse subset of neurons in the central and peripheral nervous system (Oyler et al., 1989). SNAP-25 has been demonstrated to be a presynaptic membrane SNARE, a component of the presynaptic receptor complex, which in combination with syntaxin, binds the synaptic vesicle docking and fusion apparatus (Sollner et al. 1993). The importance of SNAP-25 in modulating synaptic vesicle fusion is demonstrated by the failure of cholinergic vesicle release at the neuromuscular junction resulting from Botulinum neurotoxin A and E proteolytic cleavage of SNAP-25 (Blasi et al., 1993 and Schiavo et al., 1994).

We propose that SNAP-25 is also critical to the targeting and fusion of vesicles containing membrane and vesicle components necessary for the developmental elongation and structural maintenance of axons. Evidence for this hypothesis includes: 1. the presence of a developmentally regulated, embryonic 27 kD isoform of SNAP-25 which predominates in the brain prior to extensive synapse formation (Oyler et al., 1991); 2. the presence of intense SNAP-25 immunoreactivity in axonal fibers of embryonic and postnatal brain but not in adult brain (Oyler et al., 1992); 3. inhibition of neurite extension in PC-12 cells by SNAP-25 antisense oligonucleotides (Osen-Sand et al., 1993); and the present demonstration that axonal fibers innervating sweat glands and epidermal sensory fibers contain SNAP-25 both at presynaptic terminals as demonstrated by colocalization with synaptophysin and in axons where there was no synaptophysin immunoreactivity. The axons of sudomotor fibers and sensory fibers of skin must be frequently regenerated and actively maintained because of natural desquamation. We interpret the presence of SNAP-25 in axonal fibers of the skin apart from presynaptic terminals to be important in the maintenance of these regenerating axons.

Oyler GA et al. (1989) J. Cell Biol. 267:10613-10619., Sollner et al., (1993) Nature 362:318-324. Blasi et al. (1993) Nature 265:160-163. Schiavo et al. (1994) J. Biol. Chem. 268:23784-23787. Oyler et al. (1991) PNAS. 88:5247-5251. Oyler et al. (1992) Dev. Br. Res. 65:133-146. Osen-Sand et al. (1993) Nature. 364:445-448.

P2.17

ASTROCYTIC CELLS AT THE NEUROMUSCULAR SYNAPSE. Charlton, M.P., Robitaille, R., and Georgiou, J. Dept. of Physiology, University of Toronto, Toronto, Canada, M5S 1A8.

Astrocytes in the CNS are closely associated with synapses and respond to neurotransmitters. A glial cell at the frog neuromuscular junction, the perisynaptic Schwann cell (PSC), has several astrocyte-like properties. For instance, PSCs cover the entire nerve terminal and respond to nerve-evoked transmitter secretion with release of Ca^{2+} from intracellular stores. This can be mimicked by local application of the co-transmitters ACh or ATP (Jahromi et al., 1992; Neuron 8, 1069-1077). ACh binds to a muscarinic cholinergic receptor on these teloglia, while ATP is hydrolysed to adenosine and targets both ATP and adenosine receptors. Thus, PSCs monitor and may modulate synaptic transmission.

In the brain, the astrocyte marker glial fibrillary acidic protein (GFAP) increases with injury and activity blockade. At the neuromuscular junction, only a small proportion (11 ± 4 %) of PSCs normally express GFAP, but 6 hours after blockade of transmitter release, most PSCs (86 ± 9 %) are GFAP positive (Georgiou et al., 1994; Neuron 12, 443-455). Thus, presynaptic release of transmitter may be a signal which serves to regulate both astrocyte and PSC genes. We describe here our attempts to determine the mechanism of GFAP regulation.

Preparations were loaded with the fluorescent Ca^{2+} indicator fluo-3 AM for 1.5 hours. Either muscarine or ATP was applied every 15-30 minutes for 4.5 hours, using pressure puffs from a microelectrode positioned near PSCs. Resulting Ca^{2+} signals were observed with a confocal microscope. Preparations were then fixed and assessed for GFAP with immunocytochemistry.

Ca^{2+} signals in individual PSCs occurred for the first 1-4 puffs of muscarine, while non-desensitizing responses were seen with ATP. While both agonists activate G-protein-linked receptors, only muscarine prevented GFAP up-regulation at the puff site. PSCs elsewhere on the same preparation (>2 muscle fibres away) expressed GFAP.

We conclude that ACh, operating through muscarinic receptors, is a neuron to glia signaling molecule which prevents GFAP up-regulation in PSCs. ATP and hydrolysed products such as adenosine induced Ca^{2+} signals, but failed to prevent GFAP up-regulation, indicating that Ca^{2+} alone can not regulate GFAP and activation of the correct G-protein may also be necessary.

P2.18

NEUROMUSCULAR SYNAPSE MATURATION BY SCHWANN CELLS. IN VIVO AND IN VITRO STUDY. Koenig, J., Chapron, J., and De la Porte, S. Neurobiologie Cellulaire, Université Bordeaux II, URA CNRS 1126, Av. des Facultés, 33405 Talence Cedex - France

Using a monoclonal antibody (6.17) prepared in our laboratory, we have detected at the neuromuscular junction a new molecule which is colocalized with the acetylcholine receptor and which may be synthesized by Schwann cells. We have previously shown that: 1) in vivo in the adult rat this molecule is initially distributed diffusely and concentrates at the synapse 15 days after birth. Its maintenance at the neuromuscular junction is innervation dependent (Koenig et al., *Neurosc. Lett.*, 89, p.265, 1988); 2) during in vitro synaptogenesis, the addition of Schwann cells to neuron-muscle cocultures allows visualization of antigen 6.17 at the synapse and induces synaptic maturation (decrease in number of synapses and appearance of junctional folds). This maturation is inhibited by the presence of antibody 6.17 (Chapron and Koenig, *Neurosc. Lett.*, 106, p.19, 1989). These observations suggest that the Schwann cell may play an antigen 6.17-mediated role in maturation of the neuromuscular synapse.

The present study puts forward two new arguments in favour of this hypothesis: 1) in vitro, synaptic cholinesterase, which is initially embryonic, i.e. heterogeneous (comprising both butyryl- and acetylcholinesterase), develops into the homogeneous adult form (acetylcholinesterase) when Schwann cells are added to neuron-muscle cocultures. This effect is inhibited by the 6.17 antibody; and 2) in vivo, the butyryl- and acetylcholinesterase forms are initially present in equal amounts in the synapse of the newborn, but butyryl-cholinesterase levels diminish sharply on and after the day 15 post-natal, the stage at which the synaptic Schwann cell contacts the muscle fibre (Desaky and Ueharf, 1987).

Finally, we observed that Schwann cells greatly increased the number of AChR clusters in cultured muscle cells, without any involvement of antigen 6.17.

P2.19 β -DYSTROGLYCAN AND UTROPHIN HAVE VERY SIMILAR DISTRIBUTIONS TO THAT OF ACETYLCHOLINE RECEPTOR CLUSTERS IN CULTURED RAT MYOTUBES
Bewick G.S., Brown A., Young C. and Slater C.R. Div. of Neurobiology, University of Newcastle upon Tyne and Muscular Dystrophy Group Research Laboratories, Newcastle General Hospital, Newcastle upon Tyne, U.K.

β -dystroglycan (43K dystrophin-associated glycoprotein) is concentrated at adult mammalian neuromuscular junctions (NMJs) (Mastumura *et al*, 1992; Bewick *et al*, 1993) and is present at an early stage during synaptogenesis (see accompanying poster). Recent evidence suggests that a complex containing α and β -dystroglycan may be a receptor for agrin and, therefore, mediate nerve agrin-induced acetylcholine receptor (AChR) clustering during synaptogenesis (Sealock & Froehner, 1994). If this is so, β -dystroglycan might be expected to depend on the presence of innervation, and hence nerve agrin, to accumulate at AChR clusters. We have tested this possibility by examining the relationship between β -dystroglycan and AChR clusters formed in the absence of innervation, on the surface of rat myotubes in primary culture. We have also examined the distribution of utrophin and dystrophin, to which β -dystroglycan binds (Suzuki *et al*, 1994), relative to AChRs under the same conditions.

Highly specific monoclonal antibodies (mAbs) to β -dystroglycan, utrophin and dystrophin were used to label lightly fixed and permeabilised primary myotube cultures of rat myotubes. Labelling with the mAb to β -dystroglycan, detected with fluorescently-tagged second antibodies, was concentrated at AChR clusters, labelled with fluorescent α -bungarotoxin. Within the clusters, the distribution of β -dystroglycan labelling was also very similar to that of the AChRs. The pattern of utrophin labelling was indistinguishable from that of the AChRs. Dystrophin labelling, however, tended to be decreased in areas of high AChR concentration.

Our results show that neural influences, for example the presence of nerve agrin, are not required for β -dystroglycan to accumulate at AChR clusters. They also suggest that most β -dystroglycan at AChR clusters formed in culture is co-distributed with utrophin rather than dystrophin.

Bewick G.S. *et al* (1993) *Neuromusc. Disord.* 3:503-506. Matsumura K. *et al* (1992) *Nature* 360:588-591.
Sealock R. & Froehner S.C. (1994) *Cell* 77:617-619. Suzuki A. *et al* (1994) *Eur. J. Biochem.* In Press.

*Third International Symposium
The Cholinergic Synapse:
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UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

**POSTER SESSION 3:
(P3.1-P3.16)**

RECEPTOR REGULATION AND MODULATION

- P3.1 Calcium dependent modulation of postsynaptic nicotinic acetylcholine receptors of rat sympathetic cervical ganglia. Miu, P., Amador, M., and Dani, J.A. Division of Neuroscience, Baylor College of Medicine, Houston, Texas 77030-3498.

Recent findings have shown that the neuronal nicotinic acetylcholine receptors (NnAChRs) are modulated by external Ca^{2+} in a concentration-dependent fashion (Mulle *et al*, 1992, *Neuron* 8:937; Verino *et al*, 1992, *Neuron* 8:127). This effect can be very crucial in regulating normal nicotinic synaptic activity because high synaptic activity (e.g., repetitive stimulation of afferent inputs to nicotinic neurons) can produce millimolar reduction in external Ca^{2+} (Benninger *et al*, 1980, *Brain Res.*, 187:165; Pumain and Heinemann, 1985, *J. Neurophysiol.* 53:1) and, thus, decrease nicotinic responses. Hence the effect of external Ca^{2+} could play an important role in cholinergic synaptic plasticity. We report a possible mechanism for Ca^{2+} -mediated synaptic plasticity that differs from the intracellular Ca^{2+} cascade associated with plasticity of glutamatergic synapses. Rapid changes in external Ca^{2+} modulate both whole cell nicotinic responses in a voltage independent manner and spontaneous synaptic currents in cultured superior cervical sympathetic (SCG) neurons. Single channel conductance obtained from SCG neurons indicated that Ca^{2+} directly alters the probability of postsynaptic NnAChRs being open. Inhibition of cholinergic currents by chlorisondamine, which blocks only open channels and becomes trapped in the pore, showed that the modulation is not by a mechanism that activates a previously unresponsive population of NnAChRs. Taken together the results indicate that activity-dependent decreases in external Ca^{2+} , which occur throughout the nervous system, could directly underlie a rapid negative-feedback mechanism that decreases the postsynaptic response at cholinergic synapses. When external Ca^{2+} is decreased, presynaptic Ca^{2+} currents and transmitter release also are diminished. Thus, these presynaptic and postsynaptic mechanisms could combine to rapidly depress highly active cholinergic synapses until the external Ca^{2+} concentration recovers. (P.M. is supported by the International Human Frontier Science Program, and J.A.D. is supported by the NIH, NINDS NS21229, and the MDA).

- P3.2 CALCIUM FLUX THROUGH INDEPENDENT PURINERGIC AND NICOTINIC LIGAND-GATED RECEPTORS
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ATP is a fast neurotransmitter in peripheral ganglia and the central nervous system. ATP receptors have been proposed to be members of the nicotinic superfamily of excitatory, ligand-gated receptor. It has been suggested that ATP and ACh may act on the same population of receptors, although they produce currents with different reversal potentials and rates of desensitization. We are interested in the calcium permeability of receptors in this superfamily, and have developed a method that combines whole-cell patch clamp and calcium indicator dye techniques to quantitate the fractional calcium flux through ligand-gated channels. Neurons were acutely dissociated from sympathetic ganglia of 3-4 week old rats and subjected to whole-cell patch clamp after 4-36 hours in culture. To first establish that Ca^{2+} was flowing through independent purinergic and nicotinic receptors and not a different conformational state of the same receptor, we used the open channel blocker chlorisondamine (Chlsdm), which remains inside the pore of closed nicotinic channels. After opening all available purinergic channels with ATP, nicotine plus Chlsdm was repeatedly applied to progressively block available nAChRs. After complete block of nAChRs, ATP currents were only $3 \pm 1.5\%$ ($n=4$) smaller than before, clearly showing that ATP and nicotine activate independent receptors. To measure calcium flux, pipettes were filled with an impermeant cation solution and 1 mM Fura-2, and 300 nM thapsigargin to block Ca^{2+} -induced Ca^{2+} release from internal stores. Rapid application of nicotine or ATP in a physiological solution of 150 mM Na^+ and 2.5 mM Ca^{2+} elicited large inward currents and small Ca^{2+} transients. Agonist currents in a pure Ca^{2+} solution were smaller but produced large Ca^{2+} transients. Comparing the slope of Δ free Ca^{2+} (nM)/ charge flux (pC) in physiological Ca^{2+} to that in pure Ca^{2+} solution shows that Ca^{2+} ions carry $4.7 \pm 0.2\%$ ($n=3$) of the current through nicotinic AChRs and $6.5 \pm 0.4\%$ ($n=6$) of the current through purinergic ATP receptors. Co-release of ACh and ATP from synaptic vesicles could activate nicotinic and ATP receptors on the same cell, but this synaptic activity would produce Ca^{2+} signals with different amplitudes and temporal characteristics depending on the inherent properties of these independent populations of ligand-gated receptors.

P3.3

Ca²⁺ PERMEABILITY OF α -BUNGAROTOXIN (α -BGT)-SENSITIVE HIPPOCAMPAL NICOTINIC RECEPTORS. N.G. Castro and E.X. Albuquerque. Dept. Pharmacol. Exp. Ther., Univ. Maryland Sch. Med., Baltimore MD, 21201; Lab. Mol. Pharmacol. II, Inst. Biophysics "Carlos Chagas Filho", UFRJ, Rio de Janeiro, RJ 21944, Brazil.

Intracellular levels of Ca²⁺, a second messenger in critical neurophysiological processes, can be regulated by several neurotransmitter-gated ion channels with distinct temporal characteristics and total capacities. In the present study, we determined the relative permeabilities of major conductive cations (Ca²⁺, Na⁺, and Cs⁺) through the α -BGT-sensitive nAChR channel in cultured rat hippocampal neurons, according to classical Goldman-Hodgkin-Katz (GHK) modeling. The reversal potentials (V_R) of ACh-induced, α -BGT-sensitive currents (type IA currents as per Alkon and Albuquerque, *J. Pharmacol. Exp. Ther.* 265:1455, 1993) were measured using physiological solutions of various compositions. The experiments were performed in the presence of dihydro- β -erythroidine (0.1 μ M, which blocks α 4 β 2 nAChRs) to inhibit the activation of type II currents. Permeability ratios of various ions relative to Cs⁺ were calculated independently of the intracellular medium, using a GHK equation for V_R shifts in the presence of Ca²⁺. V_Rs were corrected for liquid-junction potentials, and ion activities were used instead of concentrations. We observed that Cl⁻ does not contribute to IA currents, and that upon switching from 150 mM Cs⁺- to 150 mM Na⁺-containing external solutions, the ACh-current V_R showed a small positive shift that could be accounted for by the higher activity of Na⁺ ions, so that P_{Na}/P_{Ca} was close to the unity. Then, the Ca²⁺ permeability was investigated using Cs⁺-based external solutions containing different Ca²⁺ concentrations. The V_R of ACh currents became more positive when extracellular Ca²⁺ concentration was raised from 1 to 10 mM, with the V_R shifts yielding a P_{Ca}/P_{Na} of about 6. In similar experiments, P_{Ca}/P_{Cs} for the NMDA currents was about 10. Thus, the native α -BGT-sensitive hippocampal nAChR channel is considerably permeable to Ca²⁺, nearly as much as the NMDA channel. Although these types of nicotinic and glutamatergic channels can both mediate Ca²⁺ influx, their current-voltage relationships are quite different, suggesting non-overlapping roles in the regulation of intracellular Ca²⁺ concentration. *Support:* NIH NS25296 & ES05730; Mol. Pharmacol. Train. Prog. UMAB/UFRJ, CNPq fellowship.

P3.4

GATING PROPERTIES OF MUTANT ACETYLCHOLINE RECEPTORS. Aylwin, M.L., Filatov, G.N., and White, M.M. Dept. of Physiology, Medical College of Pennsylvania, Philadelphia, PA 19129.

A number of affinity labeling studies have identified several tyrosine residues in the α subunit of the nicotinic acetylcholine receptor as being in or near the ligand-binding site. Studies employing site-directed mutagenesis of these residues (α Y93, α Y190, and α Y198 (the notation used is subunit/amino acid/position in the *Torpedo* receptor/substitution)) in the mouse muscle, *Torpedo* electroplax and the α 7 neuronal acetylcholine receptor have demonstrated that substitution of phenylalanine for tyrosine results in a shift in the macroscopic dose-response curves for acetylcholine-elicited currents towards higher concentration. This decrease in apparent affinity has been ascribed to either a reduction in agonist binding affinity or a reduction in the coupling of agonist binding to ion channel opening; both mechanisms would give rise to shifts in the macroscopic dose-response curves. We have used kinetic analysis of receptor gating at the single-channel level to obtain estimates for the rate constants associated with the ligand-binding and channel opening steps for mouse muscle wild-type, α Y93F, and α Y198F receptors. The results suggest that the underlying cause of the shifts in the macroscopic dose-response curves is a reduction in acetylcholine affinity for the resting, activatable, state of the receptor, with little, if any, change in the coupling process. Furthermore, it is the association rate for agonist binding rather than the dissociation rate that is most affected by the mutations.

In order for the channel to open in response to agonist binding, a local conformational change in the ligand binding site must propagate throughout the receptor. Work from the Changeux lab on homomultimeric α 7 receptors has shown that substitution of a threonine for a leucine in the M2 domain (L251T in our nomenclature) results in a marked decrease in the EC₅₀ for activation and the rate of desensitization. They have interpreted these findings as resulting from the conversion of a desensitized state from a non-conducting to a conducting state. It is not clear if the substitution must be in all subunits in the presumably pentameric α 7 receptor. We have made the equivalent mutation in the γ subunit (γ L251T) of the mouse muscle AChR and find that the single substitution results in a decrease in EC₅₀ and desensitization rate. Further characterization of the properties of this mutant and others with the same substitution in other subunits are currently underway. Supported by NIH NS 23885 and an Established Investigatorship from the AHA.

P3.5

DESENSITIZATION BY NICOTINE OF CENTRAL ACETYLCHOLINE RECEPTORS. Lester, R.A.J. and Dani, J.A. Division of Neuroscience, Baylor College of Medicine, Houston, U.S.A.

An understanding of the behavioral effects of nicotine will require knowledge of the specific interactions between nicotine and acetylcholine receptors (nAChRs) in the central nervous system (CNS). Therefore, the activation and desensitization properties of nAChRs on acutely isolated medial habenula neurons have been examined. At negative holding potentials, whole-cell patch clamp recordings demonstrated that the threshold concentration for opening nAChR channels was ~ 100 nM and at least 2 molecules of agonist were required for activation. The EC₅₀ for nAChR activation was ~ 100 μM. At higher concentrations of nicotine (300 μM), nAChR channels desensitized extensively and rapidly, but recovered rapidly provided the application was brief (< 10 sec). Prolonged incubation (1-10 mins) of the cells in lower concentrations of nicotine (100 nM - 3 μM) caused a slowly developing but pronounced inhibition of nAChR channels. The IC₅₀ for this effect was ~ 300 nM indicating the presence of high affinity desensitized state(s) of the receptor.

Differences in the effective concentrations necessary for receptor activation and desensitization can be used to predict the overall behavior of nAChRs at given levels of agonist. Thus, low concentrations of nicotine, whilst causing little receptor activation, effectively block nAChRs by stabilizing desensitized state(s) of the receptor. These data support the idea that some aspects of maintained exposure to low concentrations of nicotine, e.g. nAChR up-regulation and tolerance, may arise from nAChR desensitization. Because of the existence of multiple subtypes of nAChRs, which differ in their affinity and desensitization properties, the outcome of receptor occupation by nicotine may vary according to the distribution of the specific receptor types within the CNS.

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P3.6

CHARACTERIZATION OF A PEPTIDE RECEPTOR PARTICIPATING IN THE MODULATION OF NEURONAL NICOTINIC AChRs. Margiotta, J.F. and Pardi, D. Department of Physiology & Biophysics, and Fishberg Research Center for Neurobiology, Mount Sinai School of Medicine, New York, NY, USA.

Neuropeptides are frequently colocalized with ACh and can modulate cholinergic synaptic transmission. We recently showed that vasoactive intestinal peptide (VIP) rapidly modulates nicotinic AChRs on dissociated chick ciliary ganglion neurons (Gurantz et al., *J. Neurosci.* 14: 3540-47, 1994). Treating the neurons with 1 μM VIP enhanced whole-cell ACh sensitivity by 52 ± 11% within 10 min, and substantially increased levels of intracellular cAMP within 2 min. The rapid effect of VIP on ACh sensitivity was similar to, but not additive with, that produced by a cAMP analog, indicating that AChR modulation by VIP requires cAMP mobilization. VIP is a member of a peptide family that also includes glucagon, secretin, and pituitary adenylate cyclase activating polypeptides (PACAP27 and PACAP38), all of which share considerable homology, and stimulate cAMP production via homologous G-protein coupled receptors. Here we show that similar maximal levels of cAMP are produced (≈200 fmol/ganglion) within 3 min when peptide receptors on ciliary ganglion neurons are activated by saturating concentrations of either VIP or PACAP38. Dose-response studies reveal markedly different EC₅₀ values for cAMP production in response to PACAP38 (0.35 ± 0.05 nM, n=3) and VIP (308 ± 56 nM, n=4) however, indicating that PACAP38 is a 900-fold more potent agonist than VIP. In accord with its high potency in mobilizing cAMP in the neurons, PACAP38 (10 nM for 10 min) also enhanced whole-cell ACh sensitivity relative to untreated controls by 42 ± 11% (n=16 neuron pairs, p<0.02), an effect that is indistinguishable from that seen previously for 1 μM VIP. The functional relevance of VIP in the ciliary ganglion is supported by the presence of VIP-like immunoreactivity in presynaptic terminal vesicles (Reiner, 1987). Using a radioimmunoassay based on a PACAP38-specific antiserum that does not recognize chicken VIP, we detected 5.0 ± 2.0 pg of PACAP38-like material per ciliary ganglion (n=3). Taken together, the findings are consistent with sources of VIP- and PACAP-like peptides in the ciliary ganglion, and with the presence of functional type I PACAP receptors on the neurons that can participate in AChR modulation. Type I PACAP receptors are widely distributed in the nervous system; their binding properties reflect a high selectivity for PACAP38 over VIP, and they are activated with 100-1000-fold higher potency by PACAP38 than by VIP. The presence of such mixed affinity peptide receptors on ciliary ganglion neurons could permit differential modulation of AChR function according to the levels of available VIP or PACAP. Supported by NIH NS24417.

- P3.7 ANALYSIS OF THE TROPHIC INFLUENCE OF TESTOSTERONE ON THE NICOTINIC RECEPTOR/IONIC CHANNEL COMPLEX (AChR) IN SKELETAL MUSCLE CULTURE. Lima-Landman, M.T.; Bielavsky, M. and Lapa, A.J. Dept. of Pharmacology, Escola Paulista de Medicina, Sao Paulo, Brazil.

Development and maintenance of skeletal muscles are under trophic influence of substances of neural and/or hormonal origin. The role of innervation on skeletal muscle fibers is better known. The hormonal influences are not clearly defined mainly because the sexual influences are not evenly exerted in all muscles. The perineal musculature of the male rat is a model of musculature under testosterone influence. This musculature is not present in the female adult rat and its differentiation is delayed in male comparatively to other muscles. After castration of the male rat the perineal musculature atrophies simultaneously to pre- and post-synaptic changes. It is not known whether testosterone is acting on the muscle fibers, on the nerve terminal or on both regions. The present experiments analysed the trophic influences of testosterone on the nicotinic receptor/ionic channel complex in myoballs obtained from cultures of perineal (P) or thigh (T) muscles from 4 days old male rats. The cultures were used 7-8 days after plating. The tissue culture procedure was basically that described by Horn and Patlak (*Proc. Natl. Acad. Sci. USA*, 77: 6930-6934, 1980). The AChR complex was studied with the patch-clamp technique in the cell-attached configuration at room temperature (22 - 25°C). The single channel recordings were done in HEPES solution, pH 7.4 with TTX (300 nM). ACh (400 nM) was added to this solution in the patch pipette. The membrane holding potential was calculated assuming -65 mV as the membrane resting potential. The amplitude of the single channel currents was increased by hyperpolarization of the membrane in a range from -85 to -265 mV. More than one channel conductance was obtained. The most prevalent conductance was 35 ± 5 pS in T and 36 ± 3 pS in P. This prevalent conductance was used to study the kinetics of the channel. The frequency of openings increased with hyperpolarization of the patch membrane in T and P myoballs and the mean channel open time was prolonged by hyperpolarization of the patch. At -165 mV the mean channel open time in P (5.1 ± 0.7 msec) was 374% longer than in T myoballs (n=4). These results indicate that the kinetics of the AChR complex is slower in myoballs from cultures of the hormone dependent skeletal muscle than in myoballs of the thigh muscle. This finding could be related to the delayed differentiation of the perineal musculature.

(FAPESP, CNPq - Brazil)

- P3.8 THE DEGRADATION RATE OF R_s ACETYLCHOLINE RECEPTORS ON MOUSE DIAPHRAGMS IS REGULATED BY THE cAMP-DEPENDENT PROTEIN KINASE Xu, R. and Salpeter, M. M. Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853, USA

The degradation rate of the slowly degrading population of acetylcholine receptors (R_s) at the neuromuscular junction is regulated by the nerve. The degradation rate of the R_s has a t_{1/2} of 8-10 day, when muscle is innervated, accelerates to a t_{1/2} of 3 days upon denervation and restabilizes when the muscle is reinnervated. This reversible modification of the degradation rate occurs while the R_s is in the post synaptic membrane and does not require the replacement of the R_s by new receptors. The neural stabilization of the R_s can be mimicked in organ culture by DB-cAMP. We examined the mechanism by which the cAMP regulates the R_s degradation rate. Denervated mouse diaphragm muscles were labeled by ¹²⁵I-BTX in vivo and 6 days later were removed to organ culture dishes. The degradation rate of the receptor was calculated by the release of radioactivity from the muscles. Daily application of 500mM DB-cAMP elevated intracellular cAMP levels and stabilized the accelerated R_s from a t_{1/2} of ~3 days to one of ~7 days. The stabilization was reverted upon removal of the DB-cAMP from the culture medium, indicating that the slowing of R_s degradation by DB-cAMP was not due to muscle damage. In addition, R_s degradation rate was not affected by either DB-cAMP or by the membrane impermeable cAMP, attesting the requirement for elevating intracellular cAMP levels. Finally, H89, a PKA inhibitor at concentrations which prevented the increase in PKA activity induced by DB-cAMP, blocked the stabilization of R_s caused by DB-cAMP (H89 alone did not have any effect on R_s degradation rate). These results show that cAMP regulates the R_s degradation rate via a PKA mediated mechanism. The relation between this pathway and innervation in the regulation of R_s stabilization remains to be established.

P3.9

SINGLE-CHANNEL KINETICS OF ACh RECEPTORS: ACTIVATION BY DIFFERENT AGONISTS, AND THE ROLE OF TYROSINES NEAR THE BINDING SITE. Chen, J., Sine, S.* and Auerbach, A. Dept. Biophysics, SUNY Buffalo and *Dept. Physiology and Biophysics, Mayo Foundation.

Two agonist molecules bind to nicotinic receptors before the channel opens effectively. We have examined the properties of wt embryonic mouse ACh receptors expressed in HEK 293 cells, and activated by the agonists ACh, tetramethylammonium (TMA), or carbachol (CCh). TMA and ACh-activated channels close at approximately the same rate ($\sim 150 \text{ s}^{-1}$), while CCh-activated channels close somewhat faster. The EC_{50} (single channel Popen measurements) for ACh, CCh, and TMA are 9, 32, and 886 μM , respectively. Channels activated by TMA open at a rate of $\sim 3000 \text{ s}^{-1}$ after becoming fully liganded. The presence of frequent and brief ($\sim 25 \mu\text{s}$) gaps in the single channel currents are inconsistent with such a slow opening rate. With ACh as the agonist, rapid perfusion studies by other labs indicate that channels open at $>60,000 \text{ s}^{-1}$. This rate is faster than expected given the $\sim 35 \mu\text{s}$ gaps that we observed in the ACh-activated single-channel record. These results lead us to suspect that some of the fast gaps reflect sojourns in a second doubly-liganded closed state that is connected to the open state.

We have also studied the activation properties of receptors with mutations at tyrosine residues that are likely to be near the binding site (αY190F , αY198F , W, S, and T; αY93F , S, and T). Of these, only αY190F had a greatly (>150 -fold) reduced opening rate. All of the mutations show a reduced association rate to the first binding site. Aromaticity seems to be an important determinant, as the effect was greatest with S and T substitutions. None of the mutants showed enhanced dissociation rates at the first site, suggesting that interactions of ACh with these tyrosines is not rate-limiting for dissociation.

P3.10

REGULATION OF TYROSINE PHOSPHORYLATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR BY THE POSTSYNAPTIC 43K PROTEIN Qu, Z.,† Doherty, C.A.,† Hoffman, P.,‡ and Huganir, R.L.†§. †Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. §Howard Hughes Medical Institute, Baltimore, Maryland, USA, ‡Molecular Neurobiology Unit, National Institutes on Aging, Baltimore, Maryland, USA.

A variety of studies have suggested that tyrosine phosphorylation plays a role in the clustering of the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction. Recent studies have shown that coexpression of a 43K synaptic peripheral membrane protein with the AChR in heterologous expression systems can induce the clustering of the AChR. In this study, we investigated whether tyrosine phosphorylation may be involved in 43K protein-induced AChR clustering. 43K-induced AChR clusters contained high levels of phosphotyrosine as detected using immunofluorescent labeling with anti-phosphotyrosine antibodies. Co-expression of the 43K protein with the AChR resulted in tyrosine phosphorylation of the β and δ subunits of the AChR. Site-specific mutagenesis of the tyrosine phosphorylation site on the β and δ subunits of the AChR eliminated the tyrosine phosphorylation of these subunits. However, the mutations did not block clustering of the AChR, nor did they eliminate the high levels of tyrosine phosphorylation that colocalized with the 43K protein-induced AChR clusters. In fact, transfection of 43K protein alone dramatically increased tyrosine phosphorylation of several cellular membrane proteins. These results suggest that tyrosine phosphorylation of membrane proteins, but not the AChR itself, may be important for 43K-induced AChR clustering.

- P3.11 PHOSPHORYLATION OF GABA_A RECEPTORS BY MULTIPLE PROTEIN KINASES. Moss, S.J.¹, McDonald, B.¹, Krishek, B.J.², Amato, A.² and Smart, T.G.² ¹MRC Laboratory of Molecular Cell Biology and Dept of Pharmacology, University College London, Gower Street, London WC1E 6BT. ²Dept of Pharmacology, The London School of Pharmacy, 29-39 Brunswick Square London WC1N 1AX

Molecular cloning has revealed a multiplicity of GABA_A receptor subunits which can be divided into subunit classes dependent on homology: $\alpha_{(1-6)}$, $\beta_{(1-3)}$, $\gamma_{(1-3)}$, $\delta_{(1)}$, and $\rho_{(2)}$. Many GABA_A receptor subunits contain within their putative intracellular domains phosphorylation sites for a number of both serine/threonine, and tyrosine protein kinases. We have analyzed the phosphorylation of both recombinant and neuronal GABA_A receptor subunits using a combination of molecular, biochemical, and electrophysiological methodologies. Biochemical analysis of the phosphorylation of the β_1 , β_2 , and β_3 subunits has revealed that a conserved serine residue within the intracellular domain of these subunits (serine 409 in the case of the murine β_1 subunit) is phosphorylated with high affinity by: PKA, PKC, PKG, and CAM KII. Likewise we have identified phosphorylation sites for both CAM KII and PKC within both the γ_2L and γ_2S subunits. The functional effects of receptor phosphorylation, have been analyzed by comparing the electrophysiological properties of wild type phosphorylated and mutant non phosphorylated GABA_A receptors. The result suggest that phosphorylation can differentially modulate GABA_A receptor function dependent on subunit composition.

- P3.12 CONDUCTANCE OF 5-HT₃ RECEPTOR LIGAND-GATED ION CHANNELS IS CONTROLLED BY PHOSPHORYLATION. van Hooft, J.A. and Vijverberg, H.P.M. Research Institute of Toxicology, Utrecht University, P.O. Box 80.176, NL-3508 TD Utrecht, The Netherlands

In cell-attached patches of N1E-115 neuroblastoma cells 1.5 μ M 5-HT₃ (in the presence of 1 mM 5-hydroxyindole to delay desensitization, Kooyman *et al.*, 1993) evokes four single channel conductance levels of 26.7 ± 2.0 pS ($n=19$), 18.3 ± 2.1 pS ($n=16$), 11.7 ± 1.8 pS ($n=3$) and 6.7 ± 2.9 pS ($n=2$). Direct transitions between the various conductance levels indicate that the four conductances are substates of the same ion channel, similar to subconductance states of other ligand-gated ion channels. Upon inside-out excision of a cell-attached patch the main conductance level is reduced to 6.5 ± 0.9 pS ($n=3$), which is close to the value previously reported in excised outside-out patches under the same ionic conditions (van Hooft *et al.*, 1994). Subsequent re-exposure of the inside-out patch to the cytosol (patch-clamping) restores the main conductance level to 21.7 ± 0.3 pS ($n=3$). Buffering of intracellular Ca²⁺ by BAPTA and inhibition of protein kinase activity by staurosporine increase the probability of occurrence of the 6 pS level and decrease that of the 27 pS level in cell-attached patches. Conversely, stimulation of protein kinase activity by the phorbol ester PMA enhances the probability of occurrence of the 27 pS level and decreases that of low conductance levels. We conclude that phosphorylation controls the conductance of 5-HT₃ receptor ligand-gated ion channels. Since phosphorylation sites are a common feature of the intracellular loops of ligand-gated ion channels, control of channel conductance by phosphorylation may extend to other than 5-HT₃ receptor-gated ion channels.

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van Hooft, J.A., Kooyman, A.R., Verkerk, A., van Kleef, R.G.D.M. and Vijverberg, H.P.M. (1994). *Biochem. Biophys. Res. Commun.* **199**, 227.

P3.13 MECHANISM OF INHIBITION OF THE MUSCLE NICOTINIC ACETYLCHOLINE RECEPTOR (nAChR) INVESTIGATED BY A LASER-PULSE PHOTOLYSIS TECHNIQUE. Niu, Li, Grewer, Christof, *Abood, Leo G. and Hess, George P. Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY USA

Inhibition of the nAChR by procaine, a local anesthetic, cocaine, a powerful drug of abuse, and MK-801, an anticonvulsant compound and a potent inhibitor of the NMDA receptor, has been investigated. A laser-pulse photolysis technique with a 100- μ s time resolution, in combination with a cell-flow technique with a 10-ms time resolution, has been used to determine the effects of these inhibitors on (i) the rate constant of channel opening, (ii) the rate constant of channel closing, (iii) the apparent dissociation constants of the inhibitor from the closed- and the open-channel forms. (iv) The rates with which the inhibitors bind to the closed- and open-channel forms can be estimated. These measurements allow one to discriminate between two mechanisms for the inhibition reaction: (i) the inhibitor binds in the open receptor-channel and blocks it, or (ii) the inhibitor binds to a regulatory site on the closed-channel form. The results obtained with all three inhibitors exclude a mechanism in which receptor inhibition occurs solely by the inhibitor binding to the open-channel form of the receptor.

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P3.14 TYROSINE PHOSPHORYLATION OF NMDA RECEPTOR SUBUNITS. Lau, L.-E. and Huganir, R.L. Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, MD, USA.

The N-methyl-D-aspartate (NMDA) receptor plays a critical role in the induction of several forms of neural plasticity. NMDA receptors are thought to be heteromeric complexes of at least two types of subunits, the NR1 and NR2 (NR2A-D) subunits. A variety of studies have suggested that protein phosphorylation of the NMDA receptor may be important in the regulation of its function. In this study, we investigated the possibility that NMDA receptor subunits are regulated by tyrosine phosphorylation. Using affinity-purified antibodies against the different NMDA receptor subunits (NR1, NR2B, and NR2A), we immunoprecipitated these subunits from rat cerebral cortex synaptic plasma membranes. The precipitated NMDA receptor subunits were then immunoblotted using a monoclonal anti-phosphotyrosine antibody. The NR2B subunit was phosphorylated on tyrosine residues. Tyrosine phosphorylation of NR2A was also observed when synaptic plasma membranes were incubated with ATP and tyrosine phosphatase inhibitors. In contrast, the NR1 subunit did not contain any detectable phosphotyrosine. These studies demonstrate that NMDA receptor subunits are differentially phosphorylated on tyrosine residues and suggest that the NMDA receptor is functionally regulated by protein tyrosine phosphorylation.

- P3.15** **Mg²⁺ EFFECTS ON OPEN-CHANNEL BLOCKADE OF NMDA-ACTIVATED CURRENTS BY 9-AMINOACRIDINES.** M.E. Nelson¹ and E.X. Albuquerque^{1,2}. ¹Dept. Pharmacol. Exp. Ther., Univ. Maryland Sch. Med., Baltimore, MD, USA 21201; ²Lab. Mol. Pharmacol. II, IBCCF, UFRJ, Rio de Janeiro, RJ, Brazil 21944.

Single-channel patch-clamp studies we have demonstrated that novel bis-9-aminoacridines are more potent open-channel blockers of NMDA-activated currents than 1,2,3,4-tetrahydro-9-aminoacridine (THA) in cultured rat hippocampal neurons (*Mol. Pharmacol.*, 46:151-160, 1994). Based on the acridine-induced reduction in single-channel open times at -80 mV, the blocking rate constants were found to be $1.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $1.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for 1,2-propane-bis-9,9'-aminoacridine (1,2-PAA) and 1,4-butane-9,9'-aminoacridine (1,4-BAA), respectively, compared to $3.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for THA. However, in the presence of Mg²⁺ the interaction of the 9-aminoacridines with the NMDA receptor could not be described by a mechanism of open-channel blockade, because the blockade did not follow the predictions of a simple bimolecular process, i.e., the relationship between drug concentration and channel open time in the presence of Mg²⁺ was not linear. Further testing of the effects of Mg²⁺ on the NMDA receptor single-channel kinetics showed that Mg²⁺ was not simply competing for the channel in a mutually exclusive way. Testing of models led to the suggestion that Mg²⁺ was altering the interaction of the 9-aminoacridines with the receptor, but did not prevent their binding. Additional whole-cell patch-clamp experiments were performed with a multi-barrel, fast perfusion system to study the unbinding kinetics of the 9-aminoacridines from the NMDA receptor and the effects of Mg²⁺ on this process. The recovery of the currents from blockade by the 9-aminoacridines were fit with appropriate exponential functions. In nominally Mg²⁺-free solutions, the recovery from blockade by THA had a time constant (τ_{recovery}) of $74.7 \pm 5.7 \text{ ms}$ (n=3), but this was slower than expected from the results of the single-channel analysis. The recoveries from blockade by the bis-acridines, on the other hand, were slower and had time constants of $223.4 \pm 5.9 \text{ ms}$ (n=5) and $166.5 \pm 12.2 \text{ ms}$ (n=11) for 1,2-PAA and 1,4-BAA, respectively. In the presence of $100 \mu\text{M}$ Mg²⁺ the dissociation of 1,2-PAA had a faster time constant of $122.0 \pm 14.2 \text{ ms}$ (n=4). This finding was consistent with a model of simultaneous occupancy by Mg²⁺ and 1,2-PAA (see *Mol. Pharmacol.*, 46:151-160, 1994). *Support:* USPHS Grant ES 05730.

- P3.16** **EFFECTS OF METAPHIT AND PHENCYCLIDINE (PCP) ON THE NMDA RECEPTORS OF HIPPOCAMPAL NEURONS.** Marchioro, M. and Aracava, Y. Lab. Mol. Pharmacol. II, UFRJ, Rio de Janeiro, RJ 21944, Brazil; Dept. Pharmacol. Exp. Ther., Univ. Maryland Sch. Med., Baltimore, MD 21201, USA.

Metaphit, an isothiocyanate PCP derivative, was first synthesized as a possible antagonist of PCP-induced untoward effects. Some animal behavioral studies and *in vitro* studies indicated that metaphit antagonizes PCP effects, whereas other studies suggested that metaphit mimicks PCP effects. In the present study, we studied the actions of metaphit (1-100 μM) on whole-cell currents activated by 2-sec-pulse application of an admixture of NMDA (50 μM) and glycine (10 μM) to 3-35-day-old cultured hippocampal neurons. Metaphit caused a strong voltage-dependent reduction of the peak current amplitude. At negative holding potentials, the effect of metaphit was more pronounced and practically irreversible upon wash. At positive potentials, practically no blockade was observed, and after the blockade had been induced at negative potentials, depolarization of the membrane would favor its reversal upon wash-out of metaphit. PCP induced similar voltage-dependent reduction of the peak amplitude of NMDA currents. The blocking effect of either compound appeared to be due to a reduction of the open channel probability. However, some differences were observed between the blocking actions of the two agents. For instance, PCP caused a depression of the peak amplitude and an acceleration of the decay phase of the currents, whereas metaphit caused only depression of the peak current amplitude. It is most likely that metaphit interacts preferentially with the resting state of the NMDA receptor. In agreement with our findings, PCP has been reported to interact with the agonist-bound (closed or open state) NMDA receptor. Based on our findings, we suggest that there is an allosteric, voltage-dependent site through which PCP or metaphit could exert their blocking actions. In addition, a small potentiation of NMDA-activated currents observed in the presence of metaphit or PCP suggests that both compounds have a weak partial agonist property. *Support:* FINEP & CNPq grants. *Mol. Pharmacol. Train. Prog. UFRJ/UMAB.*

*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

POSTER SESSION 4:
(P4.1-P4.23)

RECEPTOR GENE EXPRESSION

P4.1 A NEW, POWERFUL METHOD FOR THE EXPRESSION OF ION CHANNELS AND OTHER PROTEINS IN CULTURED CELLS Forsayeth¹, L.R. and Garcia², P.D., Dept. Anesthesia¹ and Pharmacology², University of California San Francisco, CA 94143-0542

We have developed a simple and efficient transfection method for transient expression of cloned genes, particularly ion channels, in cell lines and primary cultured cells. The method involves the use of DEAE-dextran to target DNA to the cellular endocytotic pathway and the use of a human adenovirus to ensure efficient lysis of endosomal vesicles. The procedure allows effective delivery of DNA into the cytoplasm and, therefore, results in a higher fraction of cells expressing exogenous proteins. Using this method, we routinely obtain approximately 90% of COS or CHO cells expressing β -galactosidase, as determined by *in situ* staining with X-gal. We have also obtained much improved levels of expression in cells that are difficult or impossible to use in transient expression assays, such as Rat-1 fibroblasts or primary osteoblast cultures. We used the method to express neuronal nicotinic receptors that require subunit assembly for proper function. The level of expression obtained was 7-10 fold higher than previously obtained. The method also proved effective to express functions in which the exogenous protein needs to couple to the endogenous cellular machinery. Thus, this transient transfection method should prove valuable for many functional studies in a broad variety of cell lines and primary cultures. This work was supported by a grant to J.R.F. from the NIH (R01-DA08373). P.D.G. is supported by the NIH Training Grant HL07731.

P4.2 PURIFICATION OF RECOMBINANT NEUROTRANSMITTER RECEPTORS EXPRESSED IN INSECT CELLS. Green, T.P., *Lummis, S.C.R. and *Stauffer, K.A. Centre for Protein Engineering and *Laboratory of Molecular Biology, MRC Centre, Cambridge, U.K.

The low abundance of neurotransmitter-gated ion channels *in vivo* makes them ideal candidates for recombinant expression. Bacterial and yeast systems, however, rarely express integral membrane proteins in significant amounts, and mammalian cell expression is not amenable to scale-up. Expression in insect cells, using recombinant baculovirus, has proved to be a good alternative, producing membrane proteins at levels up to 10 mg protein per litre of culture (mg/l)(for gap junctions). This system has been used by us to overexpress two receptor subunits; the AMPA-selective GluR1 subunit, and the 5-HT₃ receptor subunit. Both of these are known to form active homomeric channels when expressed heterologously.

For the GluR1 receptor, very high expression levels have been achieved, with over 20 mg/l produced in Sf9 cells. The receptor has proved, however, to be very difficult to solubilise, as is the case with native ionotropic glutamate receptors. In the case of the 5-HT₃ receptor, expression is more modest (~0.5 mg/l), but the use of a previously developed purification protocol has enabled us to purify the receptor to homogeneity. The structure of both receptors is being investigated by electron microscopy, and crystallisation trials for the 5-HT₃ receptor have begun. Results from the structural investigation of the two receptors will be presented.

This work was supported in part by a Zeneca/Glaxo/MRC LINK grant.

P4.3

CHANGES IN GABA_A/BENZODIAZEPINE RECEPTOR SUBUNIT mRNA EXPRESSION IN THE DEVELOPING INFERIOR OLIVARY NUCLEUS. C.C. Anne Chang, Vera Luntz-Leybman, James F. Evans, Andrej Rotter and Adrienne Frosthalm. Department of Pharmacology and the Neuroscience Program, The Ohio State University, Columbus, Ohio.

The pharmacological and physiological properties of ligand-gated ion channels are dependent on their subunit composition; spontaneously occurring changes in subunit composition during neuronal development may result in dramatic functional differences between embryonic and adult forms of the receptor complex. In the present study, *in situ* hybridization with antisense cRNA probes was used to examine the subunit composition of the gamma aminobutyric acid_A/benzodiazepine (GABA_A/BZ) receptor in the developing inferior olivary nucleus. This receptor is thought to be a pentameric chloride channel comprised of selected α , β , γ , δ and ρ subunits, the majority of which have several isoforms: α 1-6, β 1-4, γ 1-4 and ρ 1-2. Of the thirteen subunit variants present in the mammalian central nervous system, only the α 2-5, β 3, γ 1-2 subunit mRNAs are expressed at significant levels in the inferior olivary nucleus. Two clearly different temporal patterns of subunit mRNA expression were observed in olivary neurons: The α 3, α 5, β 3 and γ 2 subunit mRNAs were present during perinatal development, and became downregulated during postnatal weeks one through three. Conversely, the expression of α 2, α 4 and γ 1 subunit mRNAs was very low, or absent, during early postnatal development, but increased rapidly during postnatal weeks one and two. The presence of high concentrations of [³H]muscimol binding sites during embryonic and early postnatal development indicated that the β 3 mRNA was translated into a functional subunit. After the initial high expression, both β 3 mRNA and [³H]muscimol binding sites decreased in parallel. The benzodiazepine ligand, [³H]flunitrazepam, which binds to α 5 subunit-containing receptor complexes with low affinity, was present at low levels in the neonatal olive; the signal became further downregulated to background levels, following a similar time course to that of the α 5 subunit mRNA. These studies suggest that there are developmental changes in the subunit composition of the GABA_A/BZ receptor in inferior olivary neurons. There is accumulating evidence suggesting that, during perinatal development, GABA acts as a neurotrophic factor, an effect mediated by GABA_A receptors. The observed changes in subunit expression, which occur during a period of major alterations in afferent and efferent synaptic connections, may subserve a change in the role of GABA from its function as a neurotrophic factor to that of an inhibitory neurotransmitter.

P4.4

MUTATION OF A SINGLE AMINO ACID RESIDUE IN THE HUMAN GLYCINE RECEPTOR TRANSFORMS β -ALANINE AND TAURINE FROM AGONISTS INTO COMPETITIVE ANTAGONISTS. Schofield, P.R.*, Ragendra, S.*, Lynch, J.W.*, Pierce, K.D.*, French, C.R.* and Barry, P.H.* *The Garvan Institute of Medical Research, 384 Victoria Street, Sydney, 2010, Australia; *School of Physiology and Pharmacology, University of New South Wales, Sydney, 2052, Australia.

Missense mutations in the α 1 subunit of the glycine receptor (GlyR) which result in the substitution of Leu or Gln for Arg 271 have been identified in individuals affected by the autosomal dominant neurological disorder, familial startle disease (hyperekplexia). When expressed as homomeric α 1 subunit GlyRs, these mutations produce dramatic decreases in both glycine-binding affinity and the sensitivity of the glycine-activated chloride currents. Although the glycinergic agonists β -alanine and taurine bind to the mutant GlyRs with higher apparent affinities than glycine, their application fails to elicit a detectable current in the mutant GlyRs. Co-application of β -alanine or taurine with glycine to the mutant GlyRs, however, does result in a dose-dependent inhibition of glycine-activated currents. Comparison of glycine dose-response curves generated in the absence and presence of either β -alanine or taurine reveals that this antagonism is competitive in nature. Moreover, in contrast to glycine, prior application of either β -alanine or taurine does not alter the size of glycine-activated currents in the mutant receptors, indicating that the binding of these two compounds does not result in desensitisation of the receptor. The transformation of β -alanine and taurine from agonists into competitive antagonists is consistent with a decoupling of agonist binding and channel activation processes. The startle disease mutations have thus identified Arg 271, located at the extracellular border of the channel region, as a critical element in the mechanism that transduces these two processes in the GlyR.

P4.5 TRANSMEMBRANE TOPOLOGY OF AMPA/KAINATE RECEPTOR SUBUNITS REVEALED BY POST-TRANSLATIONAL MODIFICATIONS. Roche, K.W., Blackstone, C.D., Bernhardt, J.P., and Haganir, R.L., Department of Neuroscience, Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

AMPA/kainate glutamate receptors are oligomeric complexes composed of homologous subunits (GluR1-7; KA 1-2). The initial proposed transmembrane topology of these subunits included four transmembrane domains with the N and C termini located extracellularly. This model was largely conjectural, based on hydrophobicity analyses and comparison to the proposed topology of other ligand-gated ion channel subunits. In order to determine which regions of the AMPA/kainate subunits are intracellular and which are extracellular, we have investigated both glycosylation and phosphorylation of these subunits using site-directed mutagenesis. Asn 720 of the glutamate receptor subunit GluR6 is glycosylated in 293 cells transfected with the GluR6 cDNA. This provides convincing evidence that this residue is extracellular, contrary to the previously accepted topology model in which this residue was located in the major intracellular loop between TM3 and TM4. In addition to mapping glycosylation sites, phosphorylation of the AMPA receptor subunit GluR1 is being investigated to determine which regions of this receptor subunit are intracellular and therefore accessible to protein kinases. Using these strategies we have proposed a new transmembrane topology model for the ionotropic glutamate receptor subunits consisting of five transmembrane domains and the C terminus located intracellularly.

P4.6 ACETYLCHOLINE RECEPTOR SUBUNIT TRANSCRIPT LEVELS ARE DIFFERENTIALLY REGULATED IN DEVELOPING AND MATURE CHICK CILIARY GANGLION NEURONS. Levey, M.S., Brumwell, C.L., and Jacob, M.H. Worcester Fdn. for Exp. Biol., Shrewsbury, MA, U.S.A.

Cellular mechanisms that regulate neurotransmitter receptor expression in neurons are largely undefined. Nicotinic acetylcholine receptors (AChRs) mediate excitatory synaptic transmission in the chick parasympathetic ciliary ganglion (CG). AChRs in the CG are composed of $\alpha 3$, $\alpha 5$ and $\beta 4$ subunits. We have now established that $\alpha 3$, $\beta 4$ and $\alpha 5$ transcript levels are differentially regulated by innervation and target tissue interactions in developing CG neurons *in situ*. Using microsurgical techniques to remove the pre- or postganglionic tissues prior to synaptogenesis and quantitative RT-PCR, we determined that both innervation and target tissue interactions induce increases in $\alpha 3$ and $\beta 4$ transcript levels. In contrast, $\alpha 5$ transcript levels are increased by innervation, but target tissues have little effect.

The ability of the target tissue to regulate $\alpha 5$ transcript levels appears to change, however, after synaptic contact has been established. Surgical transection of the preganglionic nerve (denervation) or synaptic connections with the target (axotomy) in newly hatched chicks results in substantial decreases in $\alpha 5$, $\alpha 3$ and $\beta 4$ mRNA levels. The numbers of $\alpha 5$, $\alpha 3$ and $\beta 4$ transcript copies per neuron are reduced 2.5- 3-fold relative to contralateral control neuron values at 10 days after denervation and 5 days after axotomy. Declines in $\alpha 5$ mRNA levels in axotomized ganglia suggest that, in contrast to the lack of an effect of the target tissue on $\alpha 5$ levels early in development, expression of this transcript may become dependent on target tissues after synapse formation in the periphery. Innervation of the target muscle tissue may induce the expression or release of unique regulatory factors. Alternatively, declines in $\alpha 5$ levels may reflect a response to axonal injury. However, β -tubulin mRNA levels are not altered following axotomy or denervation. To avoid axonal injury, we are now using local application of colchicine to block fast axonal transport without disturbing the integrity of the axons. Preliminary results with $\alpha 3$ suggest that retrograde signals from the target tissue specifically regulate AChR expression in mature CG neurons.

Developmental changes in the relative levels of $\alpha 5$ and $\alpha 3$ subunit transcripts appear to correlate with alterations in the functional properties of AChRs in CG neurons. Thus, the differential regulation of AChR subunit transcript levels by cell-cell interactions may have important consequences for levels of synaptic activity. (Supported by NIH 21725 and MDA).

P4.7

DIFFERENTIAL REGULATION OF nAChRs IN SYMPATHETIC NEURONS BY A NOVEL ARIA SPLICE VARIANT. Yang, X., Devay, P., Yu, C., Kuo, Y. and Role, L.W. Center for Neurobiology and Behavior, Columbia University, P&S. 722 W 168th Street, PI Annex Room 807, NY, NY 10032.

ARIA increases the transcription of muscle type nAChR subunits (Falls et al. *Cell* 72, 801-815, 1993). The ARIA gene is alternatively spliced to encode many factors, including the heregulins, neu differentiation factors, and the glial growth factors. Our lab has cloned a novel splice variant, nARIA, which is unique throughout the N-terminal portion of the sequence, lacking the Ig domain typically upstream of the EFG-like domain. nARIA retains the juxtamembrane EGF-like domain, shown to be sufficient for receptor binding and activation of receptor tyrosine kinase activity (Wen et al. *MCB* 14, 1909-1919, 1994).

In the sympathetic nervous system, preganglionic input induces an ~15 fold increase in the rate of synthesis and insertion of new nAChRs and upregulates the expression of nAChR subunit genes. Previous studies with media conditioned by visceral or somatic motoneurons show increased numbers of new nAChRs on the surface of sympathetic neurons. *in situ* studies, using probes specific for nARIA or ARIA, reveal expression of these genes in somatic and visceral motoneurons. Expression of nARIA by Northern begins at E4 and is maximal at E8, while expression of ARIA begins at E6 and peaks at E8. Thus, the pattern and timing of nARIA expression is appropriate for a presynaptic signal mediating the input-induced enhancement of nAChR expression in sympathetic neurons.

To further test this idea we treated cultured E11 chick sympathetic neurons for 48 hours with conditioned media from COS1 cells transiently transfected with either the ARIA or nARIA cDNA. Assay of both ACh gated currents and subunit mRNA levels demonstrate differential regulation of nAChRs by nARIA versus ARIA. Specifically, nARIA significantly increases the maximal responses to 500mM ACh whereas ARIA significantly decreases the maximal responses compared to cultures treated with recombinant protein from the antisense construct. Measurement of nAChR subunit mRNA levels in E9 sympathetic neurons treated with nARIA or ARIA with quantitative RT-PCR reveals different profiles of subunit gene regulation. A 24 hour treatment with nARIA mimics the effects of innervation, upregulating $\alpha 3$, $\alpha 5$, $\alpha 7$, and $\beta 4$ levels, whereas ARIA down regulates $\alpha 3$ and $\beta 4$. These results suggest that nARIA may participate in the increase in nAChR subunit transcription induced by innervation of embryonic sympathetic neurons *in vivo* during sympathetic neuron development.

P4.8

EXPRESSION OF NEURONAL NICOTINIC RECEPTOR SUBUNITS IN MEDIAL HABENULA NEURONS MAINTAINED *IN VITRO*. Krauss, R. and Fischbach, G.D. Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA.

Neuronal Nicotinic Acetylcholine Receptor (nAChR) subunits belong to a family of closely related genes. They display discrete (sometimes overlapping) patterns of gene expression in CNS. The neuronal nAChRs are multimers containing a mixture of α and β subunits which determine their physiological and pharmacological properties. The mechanisms that regulate the expression of these receptors is poorly understood.

The Medial Habenula (MHb) expresses high levels of the neuronal nAChR subunits $\alpha 3$, $\alpha 4$, $\alpha 7$, $\beta 2$ and $\beta 4$. We studied the developmental pattern of expression of these subunit mRNAs in the MHb. Northern blot analysis showed that the mRNAs for $\alpha 3$, $\alpha 4$ and $\beta 4$ increase steadily from E19 to P21. The amount of $\alpha 7$ mRNA remained relatively unchanged until P18, then increased several fold between P18 and P21. The mRNA levels for $\beta 2$ remained constant during that same period.

To understand some of the mechanisms of that regulation we developed a culture system of MHb neurons. We have maintained these neurons up to 4 weeks in serum containing medium. The pattern of expression of the neuronal nAChR subunit mRNAs, in the cultures, was studied by Northern Blots. The mRNA levels for $\alpha 3$, $\beta 2$ and $\beta 4$ increased by the 4th day of culture, whereas the levels for $\alpha 7$ remained unchanged during a 6 day period.

A candidate molecule for regulating neuronal nAChRs is ARIA, a molecule that causes an induction of muscle nAChRs and increases the expression of the α subunit. ARIA is highly expressed in the medial septum, which innervates the MHb, and the putative ARIA receptor (HER4) is highly expressed in MHb. ARIA induced tyrosine phosphorylation of its receptor (p185) in MHb cells cultured for 2, 3 and 5 days. To study nAChR expression, cells were treated with ARIA at 2 or 5 days *in vitro* and treatment lasted for either one or five days. Treatment conditions included 4% fetal calf serum or serum free medium. Total RNA samples were analyzed by Northern Blots. We found no detectable change on the expression of $\alpha 3$, $\alpha 4$, $\alpha 7$, $\beta 2$ and $\beta 4$. A similar lack of effect was found in SCG neurons and PC12 cells.

The response to ARIA in the phosphorylation of p185, together with the absence of a detectable effect on the expression of neuronal nAChRs mRNA, suggests that ARIA may have functions other than regulation of these receptors. We have now an *in vitro* system to further test ARIA and other molecules. We also plan to lesion the input and output fibers from the MHb *in vivo* to determine the effect of cellular interactions in the regulation of neuronal nAChRs in the CNS.

P4.9

EXPRESSION OF NICOTINIC ACETYLCHOLINE RECEPTOR ISOFORMS IN THE DEVELOPING CENTRAL NERVOUS SYSTEM. Reinhardt, S. and Lobron, C., Institut für Physiologische Chemie, Johannes-Gutenberg Universität, Mainz, Germany; Alkondon, M. and Albuquerque, E., Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, USA

Nicotinic acetylcholine receptors (nAChR) of the vertebrate brain exist as homo- and heteropentamers of α and β subunits. Presently seven α ($\alpha 2$ through $\alpha 8$) and four β ($\beta 2$ through $\beta 5$) isoforms are known to exist in neuronal tissues of the rat, however, only limited information is available as to which combinations of subunits form functional channels under *in situ* conditions. Since Northern analysis and low resolution *in situ* hybridization studies have shown that neuronal nAChRs are differentially expressed in the adult rodent brain, we have started to determine by high resolution *in situ* hybridization the distribution, on the single cell level, of transcripts of selected nAChR isoforms in the rat brain. For this purpose we employed serial sections of rat brains of different developmental stages and primary cultures of cells obtained from selected regions of the rat brain. By combining the latter approach with electrophysiological studies of the same cells, we were able to identify three specific combinations of subunits that form functional channels.

Similar to the existence of an embryonic and an adult form of nAChR in muscle tissue, the expression of neuronal nAChR isoforms is also developmentally regulated. As shown by *in situ* hybridization with digoxigenin-labeled isoform-specific cDNA probes, the expression of nicotinic receptor transcripts can be detected soon after neuronal determination is initiated. Thus, at fetal day 13, mRNAs coding for $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$, and for $\beta 3$ and $\beta 4$ are expressed throughout the neuroepithelium, with particularly strong expression in germinal cells, whereas the $\beta 2$ isoform is not yet expressed. At day 15, when neural differentiation has further proceeded, selective expression patterns of the examined isoforms by neuronal cells can be shown. Transcripts of the $\beta 2$ isoform can be detected from prenatal day 18, i.e. when it is expressed together with the $\alpha 4$ isoform in hippocampal neurons (1). These data may suggest that the $\alpha 4$ isoform of neuronal nAChR only forms a functional channel in the presence of the $\beta 2$ isoform.

1) Alkondon, M., Reinhardt, S., Lobron, C., Hermesen, B., Maelicke, A. and E.X. Albuquerque; J. Pharmacol. Experim. Therap., in press

P4.10

STUDY OF AN AGE-RELATED EXPRESSION OF THE $\alpha 4$ -1-SUBUNIT mRNA OF THE NICOTINIC RECEPTOR IN THE RAT BRAIN

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Alteration of the central cholinergic system appears to be associated with memory impairments in humans as well as in animals and is an important feature in normal aging process and age-related disorders (e.g. Alzheimer's disease). A marked reduction of nicotinic binding sites of the nicotinic acetylcholine receptor (nAChR) is one of the striking age-related changes, pointing to a dysfunction of the cholinergic receptors and may therefore limit the usefulness of clinical therapies based on cholinomimetic drugs. In order to develop new strategies for treatment and to improve the knowledge of normal aging process, it is important to elucidate whether the reduced binding of nicotine may be due to an alteration of the nAChR-subunit composition, to a general decrease in expression of the nAChR or to post-translational modifications.

The expression of the $\alpha 4$ -1 subunit of the nAChR was studied in brain tissue (parietal cortex, hippocampus) from 3, 24 and 33 months old male Wistar rats. *In situ* hybridization was performed using digoxigenin-labeled *in vitro* transcripts specific for the $\alpha 4$ -1 nAChR-subunit. Hybridized probes were visualized by means of an alkaline phosphatase conjugated digoxigenin-antibody and a colour substrate reaction. There were no indications of marked age-related changes in the regional distribution pattern of the $\alpha 4$ -1 subunit expression neurons. Although the density of $\alpha 4$ -1 mRNA expressing neurons appears to be decreased in 33 months old rats, preliminary quantitative data point to a concomitant loss of parietal cortical neurons. These findings do not support the hypothesis that the $\alpha 4$ -1 subunit expression is decreased in aged rats. Studies are under way to check for the age-dependent gene expression of other neuronal nAChR subunits.

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P4.11

RECOMBINANT EXPRESSION OF HUMAN NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR SUBTYPES IN *XENOPUS* OOCYTES. Chavez-Noriega, L., Urrutia, A., and Johnson, E.C. SIBIA, Inc., 505 Coast Blvd. South, La Jolla, CA 92037.

In vitro transcripts prepared from DNAs encoding human neuronal nicotinic acetylcholine receptor combinations $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 4\beta 4$, and $\alpha 7$ were injected into *Xenopus* oocytes. Inward currents elicited by nicotinic agonists were recorded with two electrode voltage clamp. We compared the relative response to Acetylcholine (ACh), Nicotine (Nic), Cytisine (Cyt), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) and methylcarbamylcholine (MCC). We have compared our results to a similar study of the rat homologs by Luetje and Patrick (J. Neurosci 11:837-845, 1991). Human $\alpha 4\beta 2$ has essentially similar sensitivities to ACh, Nic, Cyt and DMPP as the rat homologs. However, we observed differences in the agonist sensitivities of human $\alpha 3\beta 4$, $\alpha 4\beta 4$, $\alpha 3\beta 2$ and $\alpha 7$ as compared to the corresponding rat homologs. For human $\alpha 3\beta 4$ we observed a rank order of potency of DMPP > Cyt \approx Nic \approx ACh. For human $\alpha 4\beta 4$ we observed a rank order of potency of Nic \approx ACh \geq MCC > DMPP \approx Cyt. These human $\beta 4$ -containing homologs have low sensitivity to cytisine while the rat $\beta 4$ containing receptors are most sensitive to cytisine. For human $\alpha 3\beta 2$ we observed a rank order of potency of DMPP > MCC \approx ACh \geq Nic \approx Cyt. For human $\alpha 7$ we found that the response ratio of Nic: ACh is ≤ 1 while in the rat homolog it is $\approx 2-4$. These results indicate that the human neuronal nicotinic receptors $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 4$ and possibly $\alpha 7$ are pharmacologically different from their rat homologs.

P4.12

EFFECT OF ANTAGONISTS ON THE TRANSIENT EXPRESSION LEVELS OF THE $\alpha 7$ SUBUNIT OF THE NICOTINIC RECEPTOR IN MAMMALIAN CELLS. Davy, R., Wonnacott, S., Wolstenholme, A. School of Biology and Biochemistry, University of Bath, Bath, U.K.

The $\alpha 7$ subunit of the nicotinic acetylcholine receptor forms functional homomeric channels when expressed in *Xenopus* oocytes and is exquisitely sensitive to nanomolar concentrations of the subtype-selective antagonist methyllycaconitine. Thus, this subunit is very attractive for the study of structure of the ligand-binding sites of the nicotinic receptor, but as yet the successful expression of this subunit in cultured mammalian cells has not been reported, limiting experimentation to the less convenient oocyte system. We subcloned a cDNA encoding the chick $\alpha 7$ subunit into the expression vector pRc/CMV and transfected it into COS-7 cells. The receptor was expressed at very low levels, judged by specific binding of 10nM [125 I]- α -bungarotoxin to transfected cells. Transfection in the presence of the antagonists hexamethonium bromide or *d*-tubocurarine increased the amount of specific toxin binding by up to 260%. This binding was inhibited by methyllycaconitine with an IC₅₀ of approximately 10nM. When expression was assessed by the binding of 100nM FITC-conjugated α Bgt, transfection in the presence of 10 μ M tubocurarine increased both the number of cells exhibiting specific cell surface fluorescence and the intensity of that fluorescence. Taken together, these data suggest that the presence of the antagonists increases the number of receptors expressed at the surface of transfected cells and that this might be a useful way of improving transient expression systems used for neuronal nicotinic receptors. This work was funded by an SERC studentship to R.D. and by a grant from the E.C.

P4.13

NICOTINE-INDUCED INCREASE IN NEURONAL NICOTINIC RECEPTORS RESULTS FROM A DECREASE IN THE RATE OF RECEPTOR TURNOVER

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Chronic nicotine exposure in tobacco smokers or experimental animals is known to cause an increase in brain nicotine binding sites and to cause accumulation of chronically desensitized receptors. Acetylcholine receptors of the same $(\alpha 4)_2(\beta 2)_3$ subunit composition as the predominant subtype of brain nicotinic receptor with high affinity for nicotine have been expressed in *Xenopus* oocytes and in a permanently transfected fibroblast cell line. Chronic exposure of these cells to nicotine, other agonists, or a channel blocker is shown to result in an increase in receptor amount, indicating that nicotine induced upregulation reflects properties of the $\alpha 4\beta 2$ receptor protein, rather than an adaptive response unique to the neurons in which these receptors are normally expressed. The nicotine concentration-dependence, time course, and extent were similar to those reported for receptor in brain, suggesting that this intrinsic property could account for the upregulation observed in brain. Upregulation does not appear to require ion flow through the ion channel, because it is also caused by a mecamylamine which blocks the ion channel, and because after prolonged exposure to nicotine most receptors become permanently unable to open their channels in response to binding nicotine. The noncompetitive antagonist mecamylamine blocks open channels more effectively, and so is more effective at blocking channels in the presence of nicotine. Mecamylamine and nicotine are also synergistic in causing receptor upregulation. Ligands which cause upregulation appear to induce a conformation of the receptor which is removed from the surface and degraded more slowly.

P4.14

CHARACTERIZATION OF HUMAN NEURONAL NICOTINIC ACHR SUBUNITS $\alpha 3$, $\beta 2$, $\beta 4$ AND $\alpha 5$ EXPRESSED IN *XENOPUS* OOCYTES

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We have isolated cDNAs for the human neuronal nicotinic AChR subunits $\alpha 3$, $\beta 2$, $\beta 4$ and $\alpha 5$ from a library prepared from the human neuroblastoma cell line SH-SY5Y. When co-expressed in *Xenopus* oocytes, the $\alpha 3$ and $\beta 2$ subunits form functional AChRs with an $EC_{50}=28\pm 2\mu M$ for ACh and $6.8\pm 0.5\mu M$ for nicotine, while the $\alpha 3\beta 4$ combination has an $EC_{50}=160\pm 30\mu M$ for ACh. Using radioimmunoassay, $\alpha 3\beta 2$ has a $K_d=4.95\pm 0.05nM$ for nicotine. AChRs on the oocyte surface were quantitated using ^{125}I -mAbs to $\alpha 3$ and to $\beta 2$. AChRs on the surface of oocytes were also labeled by biotin and detected by ^{125}I -streptavidin on Western blots of immunoaffinity purified AChRs. In SH-SY5Y cells, $\alpha 5$ was found associated with $\beta 2$ and on the cell surface. When expressed in oocytes, $\alpha 5$ can coassemble with $\alpha 3$, and either $\beta 2$ or $\beta 4$ subunits on the surface of cells. Although co-expression with $\alpha 5$ does not alter the pharmacological properties of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ AChRs in *Xenopus* oocytes, we do find that $\alpha 5$ decreases the surface expression of functional $\alpha 3\beta 2$ and $\alpha 3\beta 4$ AChRs. The extent of decrease is correlated with the amount of $\alpha 5$ mRNA injected. When $\alpha 5$ is co-expressed only with $\beta 2$ in oocytes, no AChR is detected on the surface, but $\alpha 5$ is found associated with $\beta 2$ in the cytoplasm, which might be part of the mechanism by which $\alpha 5$ decreases the expression of functional $\alpha 3\beta 2$ AChRs.

P4.15

FUNCTIONAL COUPLING AFTER CO-EXPRESSION OF RECOMBINANT HUMAN NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR $\alpha 3\beta 4$ SUBTYPE AND α_{1n} (N-TYPE) CALCIUM CHANNEL SUBTYPE IN HEK293 CELLS. K. Stauderman, S. Mahaffy, P. Brust, K. Elliott, K. Berckhan, S. Ellis, G. Velicelebi. SIBIA, Inc., 505 Coast Blvd. South, La Jolla, CA 92037.

Neuronal nicotinic acetylcholine receptors (nAChR) are known to conduct Ca^{2+} and Na^{+} ions. However, the relative conductance of Ca^{2+} may be small in some subtypes, making characterization of these receptors difficult using techniques designed to measure changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). One way to amplify the nAChR-mediated changes in $[Ca^{2+}]_i$ might be to couple their activation to that of voltage-activated Ca^{2+} channels. To test this concept, we transiently expressed recombinant human nAChR subtype $\alpha 3\beta 4$ in HEK293 cells stably transfected with recombinant human Ca^{2+} channels ($\alpha_{1B,2}/\alpha_{2D}/\beta_{1,3}$) sensitive to ω -conotoxin GIVA (ω -CgTx).

Using the Ca^{2+} -sensitive fluorescent dye fluo-3 to monitor $[Ca^{2+}]_i$, we demonstrated that HEK293 cells stably expressing $\alpha 3\beta 4$ NACHRs (clone 6H2) responded to nicotine stimulation with a d-tubocurarine-sensitive elevation of $[Ca^{2+}]_i$, 3-6 fold over basal, whereas KCl-depolarization was without effect. Conversely, cells stably expressing Ca^{2+} channels (subunits $\alpha_{1B,2}/\alpha_{2D}/\beta_{1,3}$; clone C1-C4) responded to KCl-depolarization with an ω -CgTx-sensitive increase of $[Ca^{2+}]_i$, 4-8 fold over basal levels, but did not respond to nicotine. In C1-C4 cells transiently expressing $\alpha 3\beta 4$ NACHRs (NACHR/C1-C4 cells), nicotine stimulated a rapid and marked elevation of $[Ca^{2+}]_i$ that was blocked 100% by d-tubocurarine, and 70-90% by ω -CgTx. The kinetics of the Ca^{2+} response to nicotine was more rapid in the NACHR/C1-C4 cells than in the 6H2 cells. However, the ω -CgTx-insensitive component of the nicotine response displayed similar kinetics to the 6H2 cells. Importantly, the relative potencies of the agonists nicotine, DMPP, and cytisine were unaltered in the NACHR/C1-C4 cells compared to the 6H2 cells. These data support the conclusion that the Ca^{2+} signal generated by activation of $\alpha 3\beta 4$ NACHRs was facilitated by Ca^{2+} entry through ω -CgTx-sensitive channels, demonstrating a functional coupling. Furthermore, the pharmacological profile of NACHRs in "coupled" and "uncoupled" (e.g. 6H2 cells) systems appears to be similar.

P4.16

IDENTIFICATION OF A NOVEL CIS-ACTING ELEMENT OF A NEURONAL ACH RECEPTOR GENE THAT INTERACTS WITH A DNA-BINDING ACTIVITY ENRICHED IN RAT BRAIN. Hu, M. and Gardner, P.D. Center for Molecular Medicine, Institute of Biotechnology, University of Texas Health Science Center, San Antonio, Texas, U.S.A.

Genes encoding neuronal nicotinic acetylcholine receptors are expressed in a temporally- and spatially-restricted manner. The molecular mechanisms underlying these restricted expression patterns remain largely unknown but, presumably involve the interactions of multiple *cis*-acting elements with *trans*-acting transcriptional regulatory factors. Here we report the identification and initial characterization of a transcriptional regulatory element present in the 5'-flanking region of the rat neuronal nicotinic acetylcholine receptor $\beta 4$ subunit gene. 5'-Deletional analysis of the $\beta 4$ promoter region indicates that deletion of a 19-base-pair region located 64 base pairs upstream of the $\beta 4$ transcription initiation site, leads to a significant (9- to 10-fold), but not complete, loss of transcriptional activity in transiently transfected cholinergic cell lines. Electrophoretic mobility shift assays using a radioactively-labeled double-stranded oligonucleotide corresponding to the 19-base-pair region indicate the presence of a DNA-binding activity in nuclear extracts prepared from either a cholinergic cell line or adult rat brain. In addition, the DNA-binding activity appears to be in markedly lower abundance in nuclear extracts prepared from adult rat kidney, heart, lung or liver. The DNA-binding activity is specific in that it can be competed by unlabeled specific oligonucleotide but not by oligonucleotides corresponding to binding sites for the transcription factors TFIIID, SP1 or AP1. Computer analysis of the 19-base-pair region with other known transcription factor-binding sites revealed no significant homologies. DNAase I footprint analysis is currently in progress and should help to delineate the precise boundaries of this apparently novel transcriptional regulatory element and provide a basis for isolating the regulatory factor(s) with which it interacts.

Supported by a grant from the National Institutes of Health (NS 30243).

P4.17

A COMBINED IN SITU HYBRIDISATION AND HISTOCHEMICAL METHOD FOR THE STUDY OF THE ENDPLATE SPECIFIC EXPRESSION OF ACETYLCHOLINE RECEPTOR (AChR) SUBUNIT mRNAs Sala, C., La Maestra, L., Lomo, T.* and Fumagalli, G. CNR Centre of Cytopharmacology, Dept Medical Pharmacology, University of Milano, Italy and * Inst of Neurophysiol, University of Oslo, Norway.

The nicotinic AChR is a pentameric membrane protein which is highly concentrated in the postsynaptic membrane of the neuromuscular junction and is almost absent in non-junctional regions of the muscle plasmamembrane. This restricted localisation is due to maintenance of AChR subunit specific mRNA expression by the nuclei clustered at the endplate, while the non-junctional nuclei are silenced by molecular events induced by muscle activity; any situation which inhibits muscle activity is, to various degrees, associated to activation of AChR mRNA expression by non-junctional nuclei. In addition, other trophic signals delivered by the nerve play a role and an endplate specific subunit, called ϵ is exclusively synthesised at the endplate. A suitable method for studying the role of muscle activity and nerve-derived trophic factors in controlling junctional AChR subunit mRNA expression, is in situ hybridisation. This morphological approach overcomes the problem of mRNA production by non-junctional nuclei which may become activated during the experimental manipulations. On the other hand, in situ hybridisation of endplate restricted mRNAs requires that neuromuscular junctions are unambiguously identified on the same section. Here we describe the use of a novel method for in situ hybridisation with radioactive oligoprobes on cryostat sections of muscles where neuromuscular junctions had been identified by a modification of the Karnowsky's procedure for the histochemical detection of acetylcholinesterase.

By this approach we have studied the expression of α , γ and ϵ subunit mRNAs in rat soleus muscles either innervated or denervated for up to 80 days or in muscles where the nerve evoked activity was inhibited by TTX for up to 33 days. The expression of the ϵ subunit mRNA was slightly increased as compared to control during the first 40 days of denervation; at longer time point, a reduction of the autoradiographic signal became apparent. In muscles where nerve activity was blocked by TTX, the AChE staining suggested that sprouting had occurred and the intensity of the in situ signal was increased. The in situ autoradiographic signal at the endplate for the α subunit mRNA was unchanged as compared to the controls in both denervated and TTX muscles; γ subunit mRNA signal was undetectable in innervated muscles and increased substantially at the endplates in denervated and TTX muscles. These data are in line with known evidences on AChR mRNA expression and indicate that the combined in situ-histochemical method is suitable for the study of gene expression at the neuromuscular junction.

P4.18

PUTATIVE SIGNAL TRANSDUCTION SYSTEMS MEDIATING ACTIVITY-DEPENDENT AND SYNAPSE-SPECIFIC EXPRESSION OF RAT MUSCLE NICOTINIC ACETYLCHOLINE RECEPTORS. Sapru, M., Adams, L., Walke, W. and Goldman, D. Department of Biological Chemistry and MHR, University of Michigan, Ann Arbor, MI 48109 USA

We have identified two signal transduction systems that differentially regulate adult- ($\alpha 2\beta\epsilon\delta$) and embryonic-type ($\alpha 2\beta\gamma\delta$) nAChR expression in muscle cells. Calcium serves as a common signalling molecule in both systems. Expression of the adult-type specific ϵ -subunit gene is specifically suppressed by calcium influx across the plasma membrane. In contrast, muscle activity or ryanodine induced calcium release from the muscle sarcoplasmic reticulum, results in preferential suppression of embryonic-type specific γ -subunit gene expression.

The decreased expression of embryonic-type nAChR genes by either muscle activity or ryanodine treatment can be blocked by increasing intracellular levels of cAMP. In contrast, suppression of adult-type nAChR specific genes, by calcium influx across the plasma membrane, appears to be mediated by protein tyrosine phosphorylation. Inhibition of protein tyrosine phosphatase activity increases ϵ -subunit gene expression and prevents its repression due to calcium influx. In addition, over-expression of protein tyrosine phosphatase activity specifically suppress adult- but not embryonic-type nAChR promoter activity.

Unlike that reported for chick skeletal muscle, no evidence could be found to suggest a role for calcium inducible protein kinase C activity in mediating the effects of calcium in rat muscle cell cultures. Based on the above data we propose that calcium can regulate both adult and embryonic-type nAChR gene expression. Specificity of calcium action is likely to be determined by the local expression and/or distribution of calcium-dependent signalling molecules at the sites of calcium influx (plasma membrane) and release (sarcoplasmic reticulum). Muscle activity appears to mediate its effects through a Ca^{++} /cAMP-dependent signal transduction cascade, while local expression of nAChR genes beneath the NMJ appear to be regulated by protein tyrosine phosphorylation, which is modulated by calcium influx across the plasma membrane. This work was supported by grants from NIH and MDA.

P4.19

NERVE-DEPENDENT POSITIVE AND NEGATIVE REGULATION OF AChR SUBUNIT GENE TRANSCRIPTION IN RAT MUSCLE Witzemann, V. 1), Kues, W.A. 1), Brenner, H.R. 2), Sakmann, B. 1) 1)Max-Planck-Institut für medizinische Forschung, Heidelberg, Germany; 2)Physiologisches Institut der Universität Basel, Basel, Switzerland

Innervation at the neuromuscular junction regulates the exchange of γ - and ϵ -subunits and thus determines the expression of embryonic, $\alpha 2\beta\gamma\delta$, and adult, $\alpha 2\beta\epsilon\delta$, acetylcholine receptors (AChR) of the mature endplates and also causes their preferential expression of in the synaptic region. We have used in situ hybridisation to follow changes in the spatio-temporal distribution of subunit-specific mRNAs for the rat AChR during muscle development and in denervated and pharmacologically disused muscle. Nerve-dependent signals induce at prenatal stages the exclusive subsynaptic expression of the ϵ -subunit mRNA. The α -, β -, γ -, and δ -subunit mRNAs being abundantly expressed before myoblast fusion and innervation respond to muscle activity and nerve signals during further development displaying characteristic changes in cellular distribution. In denervated adult muscle the α -, β -, γ - and δ -transcripts increase in synaptic and extrasynaptic nuclei with highest levels around synaptic regions but their distribution undergoes characteristic time-dependent changes. Block of impulse conduction by tetrodotoxin also induces a strong increase around extrasynaptic nuclei which is, however, inhibited in synaptic and perisynaptic regions. The results indicate that steady state of AChR subunit gene transcription in innervated adult muscle is regulated by both positive and negative nerve-dependent signals at the neuromuscular junction.

P4.20

INFLUENCE OF SYNTROPHIN- $\alpha 1$ ON CLUSTERING OF AChRS EXPRESSED IN COS CELLS. Yu, X.-M. and Cohen, J. B. Department of Neurobiology, Harvard Medical School, Boston, MA 02115

Syntrophin- $\alpha 1$, the 58 kD protein first identified in Torpedo electric organ, has been demonstrated to be enriched at the neuromuscular postsynaptic membranes of skeletal muscles in various species including mouse, rat, chicken and xenopus, and to be colocalized with AChR clusters in cultured myotubes. Syntrophin also exists in a dystrophin-glycoprotein complex that links cytoskeletal proteins to the extracellular matrix and includes dystroglycan- α that has recently been shown to be a functional agrin receptor. The involvement of syntrophin- $\alpha 1$ in AChR clustering is being studied by coexpression of the mouse AChR and syntrophin in COS expression system and double labeling of both immunocytochemically. Although coexpression of the 43 kD and AChR causes AChR clustering, syntrophin on its own does not form clusters or induce AChR clustering. Unlike AChRs, expressed syntrophin is not associated with clusters of expressed 43 kD protein. Studies are underway to assess the role of syntrophin and other dystrophin-family proteins on the 43 kD induced AChR clustering in COS cells.

P4.21

TRANSGENIC ENGINEERING OF NEUROMUSCULAR JUNCTIONS IN *XENOPUS LAEVIS* EMBRYOS TRANSIENTLY OVEREXPRESSION KEY CHOLINERGIC PROTEINS Michael Shapira, Shlomo Seidman, Meira Sternfeld, Rina Timberg, Daniela Kaufer, James Patrick, and Hermona Soreq Department of Biological Chemistry, Hebrew University of Jerusalem, Jerusalem 91904, Israel.

To examine the role of key cholinergic proteins in the formation of neuromuscular junctions (NMJ), we expressed DNAs encoding the mouse muscle nicotinic acetylcholine receptor (nAChR) or human brain and muscle acetylcholinesterase (hAChE), in developing *Xenopus laevis* embryos. Cytochemical staining for AChE and fluorescent labelling of α -bungarotoxin binding sites revealed expression of both transgenes in cultured myotubes from microinjected embryos. Furthermore, acetylthiocholine hydrolysis and α -bungarotoxin binding in homogenates of transgenic embryos revealed transient overexpression of the respective proteins for at least 4 days post-fertilization. Moreover, hAChE injection induced an approximately 2-fold increase in endogenous *Xenopus* nAChR. Electron microscopy coupled with cytochemical staining of AChE activities revealed that AChE-stained areas, which reached $0.17 \mu\text{m}^2$ in NMJs of control embryos raised at 21°C , increased up to 0.53 and $0.60 \mu\text{m}^2$ in nAChR and hAChE transgenics, respectively. These increases coincided with the appearance of a novel class of large NMJs, with average post-synaptic lengths up to 1.8-fold greater than controls. As much as 57 and 34% of the NMJs in animals transgenic for NACHR or hAChE, respectively, displayed AChE activity in nerve terminals in addition to muscle labeling, as compared with 10% nerve-labeled NMJs in control animals. Moreover, area, but not length values were more than 2-fold larger in hAChE-expressing NMJs labeled in their nerve terminals than in those labeled in muscle alone, reflecting hAChE-induced increase in synaptic cleft width. These findings indicate that modulation of cholinergic neurotransmission in NMJs modifies the features of nerve-muscle connections.

P4.22

VARIATION IN EGF-LIKE DOMAINS OF MEMBERS OF THE ARIA FAMILY IN CENTRAL NERVOUS SYSTEM AND SKELETAL MUSCLE. Rosen, K.M. and Fischbach, G.D. Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA.

A protein that we have isolated and cloned from chicken brain known as ARIA (Acetylcholine Receptor Inducing Activity), has been shown to be a member of a large family of related molecules that are likely to be alternatively spliced products of one gene. One structural domain of this molecule, the EGF-like domain, has received considerable attention because of its ability to produce biological activity when it alone is expressed by recombinant DNA methods. This region has also been of keen interest because it is the site of considerable sequence variation between the various family members and, in fact, forms the basis for the original classification of these related ligands as being either α or β forms.

Using nuclease protection, PCR based and other techniques we have been examining EGF-like domains in ARIA-related RNAs in the central nervous system. We have characterized RNA from numerous embryonic and early postnatal aged animals and have found that several possible EGF-like domains are utilized in the CNS. In both spinal cord and brain, the $\beta 1$ isoform appears to be the most abundant, but it is not the sole form utilized in either of these regions. Since preliminary experiments have indicated that the β isoforms of EGF-like domains are much more potent than the α in our acetylcholine receptor bioassay, we are currently trying to understand the overall functional significance of this variation, especially in light of the other structural variations known to exist in this family of molecules. Surprisingly, the examination of RNA isolated from chick skeletal muscle identified the presence of ARIA-related transcripts. After making this finding, we included skeletal muscle RNA in our characterization of the EGF-like domain and its range of variation. Skeletal muscle appears to express the range of EGF-like domains seen in the nervous system but utilizes them in differing abundance. We have also identified an α type domain in skeletal muscle while all attempts to date have failed to demonstrate the presence of this isoform in the CNS. Similarly, the finding of ARIA-related mRNAs in skeletal muscle raises additional intriguing questions, since previous studies in our laboratory have failed to identify any ARIA activity in muscle extracts. Our present work is focused on determining how structural variations in this family of ligands affects their capacity to act as inducers of acetylcholine receptor gene expression.

LOCALIZATION OF ACETYLCHOLINE RECEPTOR SUBUNIT mRNAs BY IN SITU HYBRIDIZATION TO STAINED ENDPLATES OF MUSCLE. Enchi Liu & Miriam M. Salpeter* Section of Neurobiology & Behavior, Cornell University, Ithaca, NY, USA.

The Karnovsky and Roots method of staining sites of acetylcholinesterase (AChE) activity at neuromuscular junctions (nmjs) has been modified to survive the lengthy, multiple steps of in situ hybridization and autoradiography. When the original method of Karnovsky and Roots is used to identify the postsynaptic muscle endplates, the stain does not survive the in situ hybridization procedures, and the localization of endplate specific mRNAs has had to rely on an indirect method whereby the location of the endplates were first documented by photography (after staining) and later matched up with grains detected the final autoradiogram. This method, however, is tedious, indirect and can lead to both false negative and false positive identification of labeled endplates. The successful modification involves secondary staining with diaminobenzidine and hydrogen peroxide using the Karnovsky-Roots staining reaction product as a catalyst.

Mounted longitudinal cryosections of mouse sternomastoid muscle were fixed and stained in one step on the slide with paraformaldehyde plus the Karnovsky-Roots stain and was followed by diaminobenzidine/hydrogen peroxide secondary staining. The tissues were then processed for in situ hybridization and probed for either the acetylcholine receptor (AChR) ϵ -subunit mRNA, known to be localized at the nmj or probed for the AChR γ -subunit mRNA, known to increase dramatically after denervation. Autoradiograms were prepared by the flat substrate monolayer stripping film method of Salpeter and Bachman. A carbon layer, evaporated over the stained tissues, protected the emulsion (double layer of Ilford L4) from possible chemography due to the stained endplates. After exposures of ~3 weeks, the emulsion was developed with D19. By this procedure, the endplate stain was retained even after the hybridization and autoradiographic procedures, and the developed grains due to the radiolabeling of AChR subunit mRNAs are localized at readily identified endplates.

We report quantitation of AChR ϵ - and γ -subunit mRNAs at these stained endplates in innervated and denervated muscle. Supported by NIH-GM10422.

*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

POSTER SESSION 5:
(P5.1-P5.12)

CHOLINESTERASES

P5.1 REDUCTION OF ACETYLCHOLINESTERASE (AChE) mRNA IN DYSGENIC MOUSE SKELETAL MUSCLES LACKING L-TYPE CALCIUM CHANNEL RECEPTORS *Z. Luo, M. Pincon-Raymond* and P. Taylor, Department of Pharmacology-0636, University of California, San Diego, La Jolla, California 92093, U.S.A.; *Institut National de la Sante et de la Recherche Medicale, U. 153, 17, Rue du Fer-a-Moulin, 75005 Paris, France.*

Treatment of C2-C12 cells in cultures with ryanodine and L-type, but not N-type, Ca²⁺ channel antagonists blocks the differentiation-induced increase in AChE expression indicating that ryanodine-sensitive Ca²⁺ channels in sarcoplasmic reticulum and L-type Ca²⁺ channels in T-tubules of skeletal muscle link to play important roles in regulation of AChE mRNA during myogenesis (Luo *et al.*, J. Biol. Chem., submitted). Measurements of transcription rates using run-on transcription and reporter gene expression, as well as the capacity for superinduction, show the increased mRNA associated with differentiation to be due to stabilization of a labile mRNA rather than enhanced transcription. Ryanodine and the L-type Ca²⁺ channel blockers do not influence muscle fusion or the enhanced expression of the nicotinic acetylcholine receptors (nAChR) associated with fusion. To confirm the importance of this signaling pathway in regulation of AChE expression in intact skeletal muscle, we examined mRNA levels of AChE in skeletal and cardiac muscles from muscular dysgenic mice lacking the skeletal type, but not the cardiac type, L-type Ca²⁺ channel receptors. Results from RNA protection experiments indicated 50-80% reductions in AChE mRNA levels in leg muscles from new born and day 18 embryonic mutant mice as compared to control mice. Similar reductions in AChE activity were also observed. In contrast to AChE transcripts, mRNA of γ -subunit of nAChR was increased in mutant mice. However, mRNA levels and AChE activity were not altered in cardiac tissues from mutant mice. These findings provide further evidence that L-type Ca²⁺ channels play an important role in regulation of AChE expression in intact skeletal muscle. The reciprocal regulation of mRNA levels of AChE and nAChR suggests distinct mechanisms of regulation controlled by L-type Ca²⁺ channels in intact skeletal muscles (Supported by GM 18360 & GM 24437).

P5.2 REGULATION OF BUTYRYLCHOLINESTERASE IN CHICK SCHWANN CELLS. *Weiß, B. and Layer, P.G. Max-Planck-Institute for Developmental Biology, 72076 Tübingen and Institute for Zoology, Technical University, 64287 Darmstadt, Germany.*

Butyrylcholinesterase (BChE) is expressed before outgrowth of motoraxons, during regeneration of crushed peripheral nerves, and during neurological disorders including Alzheimer's disease or glioblastomas. Since a glial origin of BChE in all these cases seems likely, we here compared the relation of chick Schwann cell (SC) proliferation with their capacity to express butyrylcholinesterase *in vitro*. Schwann cells were prepared from chick sciatic nerve to establish long-term cultures. SC were enriched by a selective medium without the use of antimetotics to suppress fibroblasts. After two weeks, SC cultures spontaneously started to proliferate without stimulation by growth factors. Cultures were characterized with antibodies against HNK-1, S-100, GFAP, Gal c and BChE. Cocultures of SC with dorsal root ganglion neurons showed that SC retain normal functionality like myelination. Proliferation and expression of cholinesterases in long-term cultures were regulated by cytokines, growth factors and BChE. Most noticeably, the most potent agent to stimulate both cell proliferation and expression of BChE was BChE itself. BChE effects can be seen also in defined medium and can be counteracted by the BChE-specific inhibitor iso-OMPA, showing their high specificity. These data suggest a prominent role for BChE in SC regulation. They further support our recent findings on regulatory functions of BChE in cell proliferation, neurite outgrowth and differentiation (refs.)

Layer, P.G., Weikert, T., Alber, R. (1993), Cell Tissue Res., 273, 219-226
Layer, P.G., Willbold, E. (1994), Int. Rev. Cytol., 151, 139-181

P5.3

LOCAL MEMBRANE DEPOLARIZATION REGULATES ACETYLCHOLINESTERASE EXPRESSION IN MULTINUCLEATED MYOTUBES. S.G. Rossi, A.E. Vazquez*, and R.L. Rotundo. Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida 33136.

The expression of acetylcholinesterase (AChE) mRNA and protein is regulated in part by membrane depolarization. Treatment of tissue-cultured myotubes with tetrodotoxin (TTX) reversibly blocks accumulation of cell surface AChE, whereas agonists that maintain the membrane in a depolarized state, such as veratridine or scorpion toxin, elevate its expression. AChE catalytic subunits are translated and assembled in the vicinity of the nuclei expressing their mRNA, and the newly-synthesized molecules subsequently transported to the overlying region of the membrane (Rossi and Rotundo, 1992). These observations indicate that synthesis, targeting, and localization of AChE is compartmentalized in myotubes, and suggest the hypothesis that locally-generated signals originating at the plasma membrane overlying individual nuclei may be responsible for locally regulating AChE expression. To test this possibility, we designed culture chambers to isolate the tissue culture medium overlying small regions of individual tissue-cultured quail myotubes, and sodium channel agonists or antagonists were added to one side. The distribution of AChE clusters per nucleus was determined by indirect immunofluorescence using an anti-AChE mAb, and Hoescht 33342 to stain the nuclei. Sodium channel antagonist such as TTX suppressed the formation of cell surface AChE clusters whereas veratridine and scorpion toxin increased their appearance. When small segments of cultured myotubes were exposed to TTX, AChE cluster formation was suppressed only on that region. Conversely, scorpion toxin increased AChE cluster formation only where in contact with the muscle surface. The propagation of spontaneous contraction was inhibited only in those fiber regions exposed to TTX, and the morphology of the myotubes remained unchanged over the entire length of the fibers regardless of treatment. These results indicate that regulation of AChE by membrane depolarization is highly compartmentalized involving signals generated at cell surface domains overlying individual nuclei. Preliminary studies using *in situ* hybridization suggest that AChE mRNA levels also depend on membrane depolarization suggesting that the perinuclear expression of AChE transcripts may also depend upon signals generated on the overlying membrane. This work was supported by grants from the NIH and the MDA to RLR.

P5.4

FTIR-SPECTROSCOPIC STUDIES ON THE SECONDARY STRUCTURE AND TEMPERATURE BEHAVIOUR OF ACETYLCHOLINESTERASE

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The secondary structure of the acetylcholinesterase of *Torpedo californica* and its temperature behaviour have been investigated by Fourier-transform infrared spectroscopy. The secondary structure was determined using the spectral features observed in the amide-I band (in H₂O buffer) and amide-I'-band (in D₂O buffer). The content of secondary structure elements was estimated as 34-36% for α -helices, 19-25% for β -sheets, 15-16% for turns and 13-17% for irregular structures, which is in good agreement with the X-ray data for the crystalline protein. In conjunction with the X-ray data, two bands in the D₂O-spectra at 1648 and 1656 cm⁻¹ were assigned to different populations of long and short α -helices. In *Torpedo* AChE, a total loss of enzymatic activity is observed in the temperature range from 35 to 40°C; in this range no spectral changes are observed. Near 56°C, however, all component bands assigned to secondary structure elements disappear abruptly, while bands assigned to β -aggregation increase. The simultaneity of both these processes is indicative of the co-operativity of the unfolding of the protein.

A large part of the primary structure of AChE from the venom of the cobra *Naja naja oxiana*, including some sites of post-translational modifications, has been determined by peptide sequencing and mass spectrometry.

P5.5 BINDING OF CATIONS AND PROTONS IN THE ACTIVE SITE OF ACETYLCHOLINESTERASE.

Gilson, M.K., Antosiewicz^{1,2}, J., Wlodek, S.¹, and McCammon, J.A.¹, Center for Advanced Research in Biotechnology, 9600 Gudelsky Dr., Rockville, MD 20850. ¹Dept. of Chemistry, University of Houston, Houston, TX 77204-5641. ²On leave from Dept. of Biophysics, University of Warsaw, 02-089, Poland.

Acetylcholinesterase (AChE) possesses a catalytic triad similar to that of the serine proteases, such as chymotrypsin. It is generally believed that the active site histidine, His 440, of AChE must be neutral for catalysis to proceed. Measurements of enzyme activity as a function of pH suggest that the pKa of His 440 is ~6.3, similar to the pKa of ~7 for the catalytic histidine, His 57, of chymotrypsin.

We have recently reported a rather accurate method for predicting the pKas of ionizable groups in proteins from their three-dimensional structures (J. Antosiewicz, J.A. McCammon, M.K. Gilson, *J. Mol. Biol.* 238:415-436, 1994). In tests against ~60 measured pKas, the largest error of the method was 2.1 pKa units, and the root-mean-square error was 0.9 pKa units. Application of this method to chymotrypsin yields a pKa of ~7 for His 57, in good agreement with experiment. However, when we apply this method to AChE, we predict a pKa of ~9.3 for His 440, in marked disagreement with the experimental value of 6.3.

The chief reason the pKa of His 440 in AChE is predicted to be so different from that of His 57 of chymotrypsin is that Glu 199 lies only 4 Å from His 440. We discuss possible factors which might compensate for the effect of Glu 199 on His 440. Perhaps the most attractive explanation involves cation-binding near Glu 199: the idea that cation-binding in the active site shifts the pKa of His 440, and thus facilitates catalysis, offers explanations for some otherwise puzzling experimental data.

P5.6 ACTIVATION OF PROTEIN KINASE C IS REQUIRED FOR ACETYLCHOLINESTERASE EXPRESSION IN SKELETAL MUSCLE: INVOLVEMENT OF MUSCARINIC RECEPTORS AND DAG PRODUCTION. Godinho, R.O. and Rotundo, R.L. Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida, USA.

Regulation of acetylcholinesterase (AChE) in skeletal muscle requires signals to repress its expression in non-innervated regions while others maintain appropriate levels of synthesis at the neuromuscular synapse. These regulatory mechanisms are coupled to electromechanical activity, which affects multiple intracellular second messenger pathways, and to signals provided by the motor neurons. Previous studies from our lab indicated that activators of protein kinase C (PKC) can increase AChE activity independent of muscle contraction. To determine the mechanisms whereby nerve stimulation results in increased diacylglycerol (DAG) production, and hence PKC activation, in skeletal muscle, we studied the effects of cholinergic agonists on the production of DAG and AChE synthesis. Treatment of quail muscle cultures with carbachol stimulated DAG production ($EC_{50} = 5 \times 10^{-5}$ M), whereas inclusion of atropine or pirenzepine produced a dose-dependent inhibition ($IC_{50} = 10^{-9}$ and 8×10^{-7} M, respectively). Atropine (10^{-9} M) shifted the dose response curve of carbachol but did not change the maximal effect suggesting competitive inhibition. DAG production was stimulated by oxotremorine-M (Oxo-M) but not by nicotine indicating that this effect occurs via activation of muscarinic receptors on the myotubes. These observations were confirmed by detection of saturable specific binding of the muscarinic ligand quinuclidinyl benzilate (³H-QNB) that was blocked by 1 μM unlabeled QNB. Moreover, there was a linear relationship ($r = 0.997$) between ³H-QNB binding and inhibition of DAG production induced by Oxo-M. Treatment of TTX-paralyzed muscle cultures with Oxo-M increased production of all AChE forms, including the synapse-related collagen-tailed form, indicating that cholinergic activation of muscarinic receptors can increase AChE expression. In contrast, pretreatment of cultures with chelerythrine to inhibit PKC blocked the Oxo-M induced increase in AChE as well as reduced total AChE in unstimulated cells by 80-90%. These observations, together with studies indicating compartmentalized increases in DAG production in skeletal muscle, suggest that a highly localized activation of PKC may be in part responsible for the increased expression of AChE at the neuromuscular synapse. Studies funded by NIH grants to RLR and a FAPESP Fellowship to ROG.

P5.7

INHIBITION OF ACETYLCHOLINESTERASE IN VARIOUS REGIONS OF THE ADULT RAT BRAIN BY TACRINE, PHYSOSTIGMINE AND HUPERZINE A. Ved H.S., Dave J.R., Doctor B.P. Division of Biochemistry and Neuropsychiatry, Walter Reed Army Institute of Research, Washington, D.C. 20307, USA.

Current therapeutic strategies for the treatment of Alzheimer's Disease (AD) aim mainly to alleviate associated cognitive deficits by activating the defective cholinergic transmission. This treatment is based upon the widely accepted "cholinergic hypothesis" that the AD brain has suffered a profound loss of cortical and hippocampal cholinergic innervation, as reflected in a loss of choline acetyltransferase and other cholinergic biochemical markers. Among these targets, there has been considerable interest in the development of acetylcholinesterase (AChE) inhibitors, such as velnacrine, tacrine, amiriden, E-2020 acid, huperzine-A, etc., as potential therapeutic drugs. Huperzine A (Hup A) was recently shown to be a potent, reversible inhibitor of AChE (*Ashani et al; 1992: BBRC 184:719-26*). The fairly long half-life ($T_{0.5} = 35$ min) for AChE-Hup A complex is in marked contrast to the rapid on/off rates that characterize other reversible inhibitors of AChE with similar potency (*Taylor and Radić: Annu Rev Pharmacol Toxicol 87:281-320*). It has the potential to improve learning and retrieval processes (*Laganier et al; 1991: Neuropharmacol 30:763-68*). Secondly, brain lesions in AD patients vary in the different regions. While senile plaques are predominantly found in the cortex, morphological changes are more profound in hippocampus. In the present studies, we have observed the *in vitro* differences in the ability to inhibit AChE by Hup-A, tacrine and physostigmine in the various anatomical regions of the adult rat brain homogenates. The findings show that while all these compounds inhibited AChE in a dose-dependent manner, the degree of inhibition varied from inhibitor to inhibitor. The rank order of potency for the inhibition of whole rat brain AChE was physostigmine > Hup-A > tacrine. The AChE inhibition varied in the different brain regions. Hup-A was most effective in the cortex > hypothalamus > cerebellum > hippocampus. In contrast, tacrine was most effective in the cerebellum > hypothalamus > cortex > hippocampus, whereas physostigmine's rank order of inhibition was cerebellum > cortex > hippocampus > hypothalamus. These findings suggest that different AChE inhibitors may have varying degree of effectiveness in the distinct anatomical regions of rat brain.

P5.8

IDENTIFICATION OF AMINO ACID RESIDUES INVOLVED IN THE BINDING OF HUPERZINE A TO CHOLINESTERASES. Saxena, A¹, Qian, N², Kovach, I.M², Kozikowski, A.P³, Pang, Y.P³, Vellom, D.C⁴, Radić, Z⁴, Quinn, D⁴, Taylor, P⁴, and Doctor, B.P. ¹Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307, ²Department of Chemistry, The Catholic University of America, Washington, DC 20064, ³The Mayo Clinic, Jacksonville, FL 32224, ⁴University of California San Diego, La Jolla, CA 92093.

Huperzine A, a potential agent for therapy in Alzheimer's disease and for prophylaxis of organophosphate toxicity, has recently been characterized as a reversible inhibitor of cholinesterases. To examine the specificity of this novel compound in more detail, we have examined the interaction of the two stereoisomers of Huperzine A with cholinesterases and site-specific mutants that detail the involvement of specific amino acid residues. Inhibition of fetal bovine serum acetylcholinesterase by (-)-Huperzine A was 35-fold more potent than (+)-Huperzine A, with K_i values of 6.2 nM and 210 nM, respectively. In addition, (-)-Huperzine A was 88-fold more potent in inhibiting *Torpedo* acetylcholinesterase than (+)-Huperzine A, with K_i values of 0.25 μ M and 22 μ M, respectively. Far larger K_i values that did not differ between the two stereoisomers were observed with horse and human serum butyrylcholinesterases. Mammalian acetylcholinesterase, *Torpedo* acetylcholinesterase, and mammalian butyrylcholinesterase can be distinguished by the amino acid Tyr, Phe, or Ala in the 330 position, respectively. Studies with mouse acetylcholinesterase mutants, Tyr337(330)Phe and Tyr337(330)Ala yielded a difference in reactivity that closely mimicked the native enzymes. Molecular mechanics energy minimization of the complexes formed between each of the two stereoisomers of Huperzine A and fetal bovine serum acetylcholinesterase, *Torpedo* acetylcholinesterase, or human butyrylcholinesterase also revealed that (-)-Huperzine A gave a better fit than (+)-Huperzine A and implicated Tyr337(330) in the stereoselectivity of Huperzine A.

P5.9

EFFECTS OF STRIATAL ACETYLCHOLINESTERASE INHIBITION ON BASAL AND CHOLINERGIC EVOKED DOPAMINE RELEASE. Dajas F.; Costa G.; Bonilla C.; Dajas-Bailador F. Instituto de Investigaciones Biológicas C. Estable. Div. Neuroquímica, Avda. Italia 3318, Montevideo, Uruguay.

The reciprocal regulation of acetylcholine (ACh) and dopamine (DA) neurotransmitter systems in the striatum is a well known fact. Both *in vitro* and *in vivo* studies have shown that ACh or nicotine increases dopamine release, alone or in presence of physostigmine or neostigmine. In order to study the effects of Fasciculin (FAS), a peptidergic acetylcholinesterase inhibitor (AChEi) in the central nervous system, FAS was applied through microdialysis or push pull cannula to the striatum of rats and extracellular dopamine was assessed by HPLC with electrochemical detection. FAS effects were compared with those of other AChEis like Paroxon and BW248c51. The per cent of AChE inhibition obtained varied according to the inhibitor utilized. At equimolar concentrations FAS inhibition of AChE activity was more potent than the other inhibitors.

None of the AChEis studied provoked changes in extracellular dopamine levels as measured by microdialysis or push pull techniques. ACh or nicotine applied through the cannula in basal conditions provoked an increase of extracellular dopamine. This effect was never observed when the AChEis were applied first. It is assumed that inhibition of AChE in the striatum blocked the normal nicotinic cholinergic control of dopamine release. This effect could be putatively attributed to the described antagonistic effects of AChEis on nicotinic cholinergic receptors.

Supported by IPICS (Project URU01), Uppsala, Sweden and CSIC, Republic University, Montevideo, Uruguay

P5.10

DOES THE BUTYRYLCHOLINESTERASE MUTANT G117H CATALYZE THE HYDROLYSIS OF ORGANOPHOSPHORUS ANTICHOLINESTERASE INHIBITORS? C.B. Millard, O. Lockridge, T.L. Caviston and C.A. Broomfield. U.S. Army Medical Research Institute of Chemical Defense, APG, MD 21010-5425 and University of Nebraska Medical Center, Eppley Cancer Institute, Omaha, NE 68198-6805

Our goal is to design, express and characterize mutants of cholinesterases that resist or hydrolyze the organophosphorous (OP) nerve agents, especially soman. Our initial studies have been concentrated on human serum butyrylcholinesterase (BuChE; EC 3.1.1.8) because of its relatively open active site region. By computer-aided molecular modeling based upon the crystal structure of acetylcholinesterase, several residues were selected for site-specific replacement with histidine. We reasoned that introducing an appropriately-positioned imidazole group could promote general base catalysis to hydrolyze the phosphorylated active site serine. The approach was oligonucleotide-directed mutagenesis in M13mp19 and subsequent stable expression in both CHO and human 293 cells by using a cytomegalovirus promoter and the geneticin drug resistance gene. One of the histidine mutants, G117H, was found to retain butyrylthiocholine (BuSCh), acetylthiocholine and benzoylcholine (Bz) activity at pH 7.4 with a $K_{m2}=0.23\pm0.017$ mM for BuSCh. Unmutated, recombinant BuChE had a $K_{m2}=0.20\pm0.016$ mM for BuSCh. Using BuSCh to measure activity, we found the inhibition rates for the BuChE G117H mutant were altered for soman, sarin, DFP and echothiophate (EcSH). For soman, sarin and DFP inhibition, unmutated BuChE showed an apparent, first-order $k_i > 2.8 \text{ min}^{-1}$ and for EcSH a k_i of 0.23 min^{-1} . However, for G117H the k_i was 0.02 min^{-1} for soman, 0.016 min^{-1} for sarin and no inhibition was found for up to 4 hr in the presence of excess ($0.24 \mu\text{M}$) EcSH. Assays of the mutant inhibited by DFP produced curved lines that indicated rapid, spontaneous reactivation and precluded determination of an accurate inhibition rate constant. ^3H -DFP was hydrolyzed significantly faster in the presence of the G117H mutant than in the presence of the unmutated BuChE. By developing a competitive ELISA for BuChE, we estimated the specific Bz activity of G117H to be $180\pm20 \text{ U/mg}$, which compares favorably to pure serum BuChE. We conclude that G117H is an active cholinesterase with unusual resistance to nerve agent inhibition and with a limited ability to catalyze the hydrolysis of DFP.

P5.11 EFFECTS OF ALDICARB, AN ANTICHOLINESTERASE INHIBITOR, ON MUSCLE NICOTINIC RECEPTORS. Braga, M.F.M., Almeida, L.E.F., Almeida, A.L., França, L.G., Côrtes, W.S., Cintra, W.M., and Aracava, Y. Lab. Mol. Pharmacol. II, IBCCF, UFRJ, Rio de Janeiro, RJ 21944, Brazil; Dept. Pharmacol. Exp. Ther., Univ. Maryland Sch. Med., Baltimore, MD 21201, USA.

Aldicarb (ALD) is a carbamate-oxime with potent anticholinesterase activity. Previous studies have demonstrated that ALD induces a concentration-dependent potentiation followed by a blockage of nerve-elicited muscle twitch at the frog neuromuscular junction (*Soc. Neurosci. Abs.* 19:1526, 1993). Because some of ALD's effects could not be fully explained by cholinesterase (ChE) inhibition, we decided to investigate whether ALD has any direct effects on muscle nicotinic receptors (nAChRs). We studied the effects of ALD on muscle contracture elicited by exogenously applied ACh or by ChE-resistant nicotinic agonists, and on ACh-evoked whole-cell and single-channel currents in preparations with negligible ChE activity. At the frog rectus abdominis, ALD (10-800 μ M) potentiated contracture tension elicited by ACh (0.2-10 μ M), carbamylcholine (5-60 μ M), or (+)-anatoxin-a (0.1-1.0 μ M). This effect of ALD was inversely related to the agonist concentration. In cultured rat myoballs, ALD (50 μ M) increased the peak amplitude of ACh (1 μ M)-evoked whole-cell currents, and in the single interosseal muscle fibers from the hind foot of *L. ocellatus*, ALD (10-100 μ M) increased the frequency of ACh (0.4 μ M)-induced single-channel activity over the recording time. On the other hand, ALD decreased the mean open time of ACh-activated channels and increased the number of brief closures within a burst. The results suggest that ALD interacts directly with the muscle nAChR, probably through an allosteric mechanism, and potentiates ACh-induced receptor activity. In addition, ALD acts as an open-channel blocker of muscle nAChR, and this blocking effect counteracts the ALD-induced potentiation of nAChR activity. *Support:* Mol. Pharmacol. Train. Prog. FINEP/UMAB; FINEP and CNPq grants and fellowships.

P5.12 ROLE FOR ACETYLCHOLINE (ACh) IN CHOLINOLYTIC CONVULSIONS Zimmer, J.A., Ennis, M., El-Etri, M., and Shipley, M.J. Department of Anatomy, University of Maryland School of Medicine, Baltimore, MD 21201

Considerable evidence suggests that cholinergic seizures are initiated in the piriform cortex (PC). Consistent with this, PC is the most frequent site of neuropathology after administration of the irreversible acetylcholinesterase inhibitor, soman, in rats and other species. We recently showed that convulsive doses of soman rapidly induce the immediate early gene protein product, Fos, in layer II-III PC neurons within 30 min. These neurons send excitatory projections to the hippocampus, thalamus, and neocortex. One hr after soman, the astrocyte specific marker, GFAP, is selectively expressed in the same layers of PC. PC is heavily innervated by cholinergic inputs from the nucleus of the diagonal band (NDB). We hypothesize that soman's anticholinesterase action may, thus, lead to cholinergic hyperstimulation of PC neurons. It is well established that ACh acting at muscarinic receptors causes postsynaptic neurons to become hyperresponsive to other excitatory inputs, especially excitatory amino acids (EAA). PC neurons receive massive EAA inputs and excite neighboring cells by EAA synapses. Thus, sustained cholinergic stimulation of PC could lead to a progressive increase in neuronal excitability, and this wave of excitability should spread throughout PC and to the hippocampus and neo-cortex. We predict that a sustained, endogenous release of ACh in PC from NDB terminals should cause seizures and selective Fos and GFAP expression in layer II-III of PC as observed following soman. To test this hypothesis, bipolar electrodes were implanted unilaterally to stimulate NDB neurons in awake, unrestrained animals. Shortly after NDB stimulation, rats exhibited behavioral convulsions and large amplitude spike and wave EEG activity; seizure-like EEG activity persisted 20-40 min after NDB stimulation ceased. Animals were sacrificed after 20 to 60 min of NDB stimulation and brain tissue was processed for immunohistochemical detection of Fos and GFAP. Consistent with our hypothesis, Fos and GFAP were selectively expressed in layer II-III of PC ipsilateral to the stimulated NDB. NDB stimulation also induced robust Fos expression in the hippocampus. Little or no Fos and GFAP expression was present in non-stimulated, control animals. Systemic administration of the cholinergic antagonist, scopolamine (2 mg/kg, i.p.) blocked the behavioral signs of NDB stimulation, and blocked or significantly attenuated the expression of Fos and GFAP in PC and the hippocampus. In a second experiment, unilateral injections of the specific cholinergic toxin *IgG saporin* were made in the NDB. After ten days, rats were subjected to a convulsive dose of soman and tissue was processed for immunocytochemical localization of Fos, GFAP, and ChAT. After 45 min, robust Fos and GFAP expression was limited to layer II-III neurons of PC on the *unlesioned* side, after 90 min, there was robust Fos and GFAP expression in PC on the *lesioned* and *unlesioned* sides. ChAT expression was eliminated unilaterally by saporin injections. These results suggest that cholinergic inputs from NDB to PC are critical for the initial induction of Fos and GFAP in PC and convulsive activity following soman. The ability of scopolamine to block NDB evoked behaviors and Fos expression indicates that these effects are mediated largely by muscarinic receptor stimulation of PC. Further studies will test this hypothesis at the cellular and membrane levels. *Supported by the U. S. Army Medical Research and Development DAMD17-91-C-1071.*

*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

POSTER SESSION 6:
(P6.1-P6.8)

NEUROTOXINS

P6.1 FURTHER CHARACTERIZATION OF HOW CEMBRANOIDS INHIBIT THE ACETYLCHOLINE RECEPTOR. Hann R.M., Lu R., Pagán O.R., Rodríguez A.D., Ferchmin P.A. and Eterović V.A. Dept. of Biochemistry and Cntr. Mol. Behav. Neurosci. U. Central del Caribe, and Dept. of Chemistry, U. of Puerto Rico Rio Piedras, USA.

Gorgonian corals contain numerous cyclic diterpenes containing the 14-carbon cembrane ring, which may act as chemical defenses against predators and competing species. Recently the laboratory of A.D.R. isolated about 20 cembranoids from Caribbean gorgonians, most of which were previously unknown compounds; their structures were determined by 2-D NMR spectroscopy.

We have recently reported that cembranoids inhibit the acetylcholine receptor (AChR) from mouse muscle. The inhibition was noncompetitive and accompanied by an increase in the rate of desensitization. In the present study, cembranoids were tested on AChR from *Torpedo californica* electric organ expressed in *Xenopus laevis* oocytes and studied with a two-electrode voltage-clamp. Eupalmerin acetate (EUAC) decreased the amplitude of ACh currents and increased the rate of receptor desensitization. The EUAC potency decreased with time after injection of mRNA into the oocyte, suggesting that a maturational process takes place with the oocyte-expressed receptor. Six additional compounds were then tested, all on the second day after mRNA injection. All cembranoids inhibited ACh-induced currents with IC_{50} 's of 0.1-35 μ M, but in every case the inhibition was incomplete. Cembranoids also inhibited 3 H-phencyclidine (PCP) binding to desensitized AChR from *Torpedo nobiliana*, with a potency rank that did not correlate with that determined in functional studies.

To further characterize the cembranoid and PCP binding sites, we used the observation that PCP displayed a sixtyfold lower potency on muscle receptor than on the electric organ receptor. Mouse/*Torpedo* hybrid receptors were expressed in oocytes and the potency of PCP and EUAC measured. Preliminary results suggested that β and γ subunits account for the difference in potency. The difference for EUAC was only threefold and the pattern observed with hybrid receptors was different from that observed for PCP. Supported by the NSF-EPSCoR and NIH-RR035.

P6.2 PROBING THE LIGAND-BINDING SITES IN THE TORPEDO ACETYLCHOLINE RECEPTOR WITH PHOTOACTIVATABLE SNAKE VENOM α -NEUROTOXINS. Utkin, Yu.N.*, Machold, J.§, Tsetlin, V.I.*, and Hucho, F.§. *Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia; §Free University of Berlin, Germany.

Three series of photoactivatable derivatives of neurotoxin II (NT-II) from *Naja naja oxiana* have been prepared. Five carbene-generating nitrodiazirine derivatives had the photolabel at Lys15, Lys25, Lys26, Lys44, or Lys46, the modified residue being identified by mass spectrometry of tryptic hydrolysates of respective derivatives. Another group of singly labeled derivatives had nitrene-generating *p*-azidobenzoyl groups at the lysine residues. A third group of Lys-derivatized NT-II analogs had an iodoazidosalicylic moiety attached via a cleavable spacer arm. With all these series, cross-linking to the α , β , γ , and/or δ subunits was detected after irradiating the complexes of the respective iodinated derivatives with the membrane-bound nicotinic acetylcholine receptor (AChR) from *Torpedo californica*. The subunit labeling patterns were dependent on the type and position of the photolabels. The results obtained demonstrate that i) not only one side of the relatively flat neurotoxin molecule is involved in the interaction with AChR, ii) two neurotoxin-binding sites of AChR have a complex configuration where fragments of different subunits are located close together. When photolabeling with Lys26-*p*-azidobenzoyl derivative, which labels the γ and δ subunits, was performed in the presence of d-tubocurarine or α -conotoxin GI, the protection of γ subunit was observed at lower concentrations of both antagonists. Hence, the (α + γ)-containing high-affinity site for d-tubocurarine is also a site of a higher affinity for α -conotoxin GI. *Acknowledgments.* This work was supported by BMT, DFG and Russian Ministry of Science.

P6.3 THE SITE PREFERENCE OF α -CONOTOXINS FOR THE TWO ACETYLCHOLINE-BINDING SITES ON THE NICOTINIC RECEPTOR IS NOT CONSERVED ACROSS SPECIES. Groebe, D.R., Dumm, J.M., and Abramson, S.N. Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261

We investigated the affinities of the two acetylcholine-binding sites on the nicotinic acetylcholine receptor for tubocurarine derivatives and the α -conotoxins in receptors from BC₃H-1 cells and *Torpedo* electric organ. The two acetylcholine-binding sites on nicotinic receptors from intact BC₃H-1 cells displayed an approximate 74-fold difference in affinity for metocurine (K_D 's of 0.35 \pm 0.1 μ M and 26 \pm 7 μ M) and an approximate 15,000-fold difference in affinity for α -conotoxin MI (K_D 's of 1.5 \pm 0.2 nM and 22 \pm 1 μ M). Dose-response curves to metocurine in the presence of 100 nM MI demonstrated that the binding site with higher affinity for MI had lower affinity for metocurine. Similar results were obtained for the α -conotoxins GI and SIA. Other investigators have previously shown that, in nicotinic receptors from BC₃H-1 cells, the γ -subunit contributes to the binding site with high affinity for tubocurarine derivatives and the δ -subunit contributes to the binding site with high affinity for MI. The two acetylcholine-binding sites from detergent-solubilized *Torpedo* electric organ membranes displayed an approximate 300-fold difference in affinity for metocurine (K_D 's of 152 \pm 43 nM and 45 \pm 10 μ M) and an approximate 660-fold difference in affinity for MI (K_D 's of 3.2 \pm 1.2 nM and 2.1 \pm 0.7 μ M). Dose-response curves to metocurine in the presence of 100 nM MI demonstrated that the binding site with higher affinity for MI also had higher affinity for metocurine. Similar results were obtained with d-tubocurarine. Other investigators have previously shown that, in nicotinic receptors from *Torpedo* electric organ, the γ -subunit contributes to the binding site with high affinity for d-tubocurarine. It appears that, in *Torpedo* electric organ, it is the γ -subunit that contributes to the binding site with high affinity for MI. The transposition of the high-affinity binding site for MI may result from subtle differences between species in the amino acid sequences of the γ - or δ -subunits despite overall sequence homology. Thus, the site preference of ligands for the two acetylcholine-binding sites present on any nicotinic receptor subtype is not necessarily conserved across species. *Supported by NIH, the Smokeless Tobacco Research Council, and the Pharmaceutical Manufacturers Association Foundation.*

P6.4 SYNERGISTIC INFLUENCE OF GLYCOSYLATION AND CERTAIN AMINO ACID SUBSTITUTIONS IN CONFERRING α -TOXIN RESISTANCE TO RECOMBINANT DNA DERIVED NICOTINIC RECEPTORS. Keller, S. H., Kreienkamp, H. J., Maeda, R., and Taylor, P. Pharmacology Department, University of California, San Diego, La Jolla, California.

Sequences of α -bungarotoxin resistant and susceptible α -subunits of the nicotinic acetylcholine receptor have been elucidated, and include substitutions encoding glycosylation at amino acids 189 (cobra) and 187 (mongoose), a substitution from proline to leucine at amino acid 194 (P194L), and an additional glycosylation signal at amino acid 111 (cobra). Our previous study using site-directed mutagenesis and mammalian cell expression of the intact receptor on the cell surface demonstrated that N-linked glycosylation signals at positions 189 and 187 conferred up to a 600 fold reduction in α -bungarotoxin affinity, whereas P194L alone had little or no effect (Kreienkamp et. al. , 1994. J. Biol. Chem. 269, 8108-8114). The mutation P194L was introduced into cDNA templates that code for the 187 and 189 glycosylation sites, and the 111 glycosylation site found in the snake was added to the template containing the 189 glycosylation site and the P194L substitution. Experiments using mobility shifts on SDS-PAGE indicate that an oligosaccharide is expressed at the 187 glycosylation site. The insertion of P194L with the 189 glycosylation site confers up to an additional 80 fold reduction in affinity, and the 111 glycosylation signal further decreases affinity two fold. Analysis of the rates of α -toxin association and dissociation suggest that glycosylation places a constraint on the receptor in producing a high affinity configuration. Supported by USPHS grand GM 24437.

P6.5

TOWARDS A MECHANISTIC EXPLANATION FOR THE IRREVERSIBLE INHIBITION OF NICOTINIC ACETYLCHOLINE RECEPTORS BY THE LOPHOTOXINS Hyde, E.G., Groebe, D.R., Xu, Y., Tang, P., Boyer, A.J., Abramson, S.N. Departments of Pharmacology, Pharmaceutical Sciences, and Anesthesiology, and the Center for Clinical Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA 15261.

Many species synthesize toxic inhibitors of voltage and ligand gated ion channels to obtain an adaptive advantage over predators or prey. Some of these toxins appear to be irreversible because they have very slow dissociation rates. Very few of these toxins, however, actually inhibit their target proteins by a covalent mechanism of action. The lophotoxins are a family of small structurally related cyclic diterpenes isolated from marine coral. These toxins irreversibly inhibit nicotinic acetylcholine receptors by interacting at the acetylcholine-recognition sites and reacting covalently with Tyr¹⁹⁰ in the α subunit of the receptor. A greater understanding of the mechanism of action of the lophotoxins may provide new insights that can be applied to the development of novel synthetic covalent site-directed affinity labels.

Although lophotoxin appears to be active by itself, several naturally occurring analogs of lophotoxin (for instance, bipinnatin A, B and C) have been shown to be inactive protoxins that are activated by preincubation in buffer. Solvolysis of bipinnatin A, B, and C in buffer was an apparently first-order process ($t_{1/2} = 1$ hour) that was relatively independent of pH, suggesting that it is not an acid or base mediated process. Incubation of these toxins in buffer resulted in the production of one mole of acetic acid per mole of toxin at a rate that was indistinguishable from the rate of solvolysis of the toxins, suggesting that solvolysis involves elimination of an acetate ester. Activated toxins produced by incubation of bipinnatin A and C in buffer were isolated by reverse-phase HPLC and their structures determined by FAB-MS and ¹H NMR. The activated bipinnatins appear to be identical to their protoxins except for replacement of an acetate ester by a free hydroxyl group. The process of activation in buffer appears to involve an S_N1 substitution reaction, where elimination of an acetate ester results in formation of a carbocation intermediate that subsequently undergoes reaction with solvent. In the lophotoxins, this carbocation intermediate is presumably stabilized by resonance with an adjacent electron-rich furan ring. Since all active lophotoxin analogs also contain an epoxide adjacent to this same furan ring, it is possible that covalent reaction between the lophotoxins and α Tyr¹⁹⁰ involves a similar S_N1 reaction at this epoxide. (Supported by NIH, the Smokeless Tobacco Research Council, and the Pharmaceutical Manufacturers Association.)

P6.6

**COMPARATIVE PHARMACOLOGY OF EPIBATIDINE,
A POTENT SELECTIVE AGONIST FOR NEURONAL ACHRS.**

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Pharmacological properties of the (+)- and (-)-isomers of the synthetic analgesic epibatidine (EB), originally isolated from the skin of the frog *Epipedobates tricolor*, were tested on different chicken and human nicotinic AChRs.

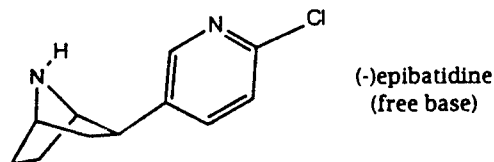
In competition binding assays EB was used on immunoaffinity isolated chicken brain ($\alpha 4\beta 2, \alpha 7, \alpha 8$) and human neuronal nicotinic ($\alpha 3$ and $\alpha 7$) AChRs (from the SHSY-5Y cell line). Both EB isomers exhibited extremely high affinity for all neuronal AChRs tested, with IC₅₀ values ranging from 1 pM (human $\alpha 3$ AChRs) to 1 μ M (chicken $\alpha 7$ AChRs). By contrast, no EB binding was observed on human muscle type AChRs from the cell line TE671.

EB behaved as an extremely potent full agonist on chicken ($\alpha 3\beta 2, \alpha 3\beta 4, \alpha 4\beta 2, \alpha 7, \alpha 8$) and human ($\alpha 3\beta 2$) neuronal AChRs expressed in *Xenopus* oocytes. Currents induced by EB were effectively blocked by the nicotinic antagonists hexamethonium and mecamylamine. Apparent affinity was 100 to 1000 times higher for EB as compared to nicotine or ACh. EC₅₀ values ranged from 1 nM (for homomeric chicken $\alpha 8$) to 2 μ M (for homomeric chicken $\alpha 7$). EB did not activate or block expressed *Torpedo* muscle type AChRs at concentrations up to 1 mM. Currents induced by EB in oocytes expressing chicken $\alpha 4\beta 2$ and $\alpha 3\beta 2$ showed significantly slower desensitization and inactivation kinetics than did currents induced by ACh and nicotine.

P6.7

EPIBATIDINE: A POTENT ANALGETIC ACTING THROUGH CENTRAL NICOTINIC RECEPTORS. Badio, B., Garraffo, H.M., Shi, D. and Daly, J.W. Laboratory of Bioorganic Chemistry, NIH, Bethesda, MD 20892.

The enantiomers of epibatidine are potent analgetics ($ED_{50} = 2 \mu\text{g}/\text{kg}$), whose central site of action appears to involve nicotinic receptors. The analgetic effects of epibatidine are blocked by mecamylamine and chlorisondamine, but not by naloxone. The L-type calcium channel activator Bay K 8644 potentiates epibatidine-elicited analgesia, while nifedipine antagonizes the analgesia. Analgetic effects of epibatidine are reduced in mice rendered tolerant to behavioral effects of nicotine by chronic treatment with nicotine.



Epibatidines are about 20-fold more potent than (-)nicotine at central [^3H]nicotine-binding sites, about 200-fold more potent as agonists at ganglionic-type receptors in PC12 cells and about 100-fold more potent as agonists at muscle-type receptors of TE-671 cells. Agonist responses to epibatidine are blocked by competitive and noncompetitive nicotinic antagonists. Epibatidine, like nicotine, causes desensitization of nicotinic receptors. Unlike nicotine, there is only slight enantioselectivity for the epibatidines either *in vivo*, with binding assays, or with cultured cells. Furthermore, the presence or absence of an *N*-methyl group has little effect on activity of epibatidines, unlike the case for nicotine/nornicotine. Molecular modeling provides a rationale for the enantioselectivity and the effects of an *N*-methyl in the case of the nictines and the lack of major effects for the epibatidines.

P6.8

IS EPIBATIDINE A SUITABLE AGONIST AT THE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS (nAChRs)? M. Alkondon and E.X. Albuquerque. Dept. Pharmacol. Exp. Ther., Univ. Maryland School of Medicine., Baltimore, MD 21201.

The diversity of nAChRs in the central nervous system (CNS) requires the search for potent, efficacious, and selective nicotinic agonists for identifying and characterizing the distinct neuronal nAChR subtypes. In this study, we used hippocampal neurons to investigate the agonist property of (-) and (+) enantiomers of epibatidine, an alkaloid isolated from the frog *Epipedobates tricolor*. This drug has been shown to bind with high affinity to [^3H]nicotine-binding sites in rat brain membranes, to induce ion fluxes in PC12 cells, and to induce analgesia in mice. Both epibatidine enantiomers could evoke type IA currents, the α -bungarotoxin-sensitive currents, with apparent EC_{50} s of $2 \mu\text{M}$ and $4 \mu\text{M}$, respectively, and evoke type II currents, the dihydro- β -erythroidine-sensitive currents, with apparent EC_{50} s of 16 nM and 10 nM , respectively. (+)Anatoxin-*a* (ANTX), another potent nicotinic agonist, elicited type IA and II currents with EC_{50} s of $4 \mu\text{M}$ and about 100 nM , respectively. (-)Nicotine, on the other hand, evoked type IA and II currents with EC_{50} s of $27 \mu\text{M}$ and about $1 \mu\text{M}$, respectively. In addition, while (-) and (+) epibatidine produced peak amplitudes close to those produced by a saturating ACh concentration in type IA currents, they elicited type II currents with amplitudes reaching about 70% and 95%, respectively, of those evoked by a nearly saturating concentration of ACh. On the other hand, the maximal type II current amplitude induced by ANTX was about 35% of that evoked by ACh, although the maximal peak amplitude of ANTX-evoked type IA currents was about 85% of that evoked by ACh. The present results indicate that: i) epibatidine is a very potent and efficacious agonist at the hippocampal nAChRs; ii) epibatidine does not exhibit stereospecificity in activating hippocampal nAChRs; and iii) epibatidine has a higher selectivity in activating type II over type IA currents than the other nicotinic agonists tested. These properties make epibatidine a unique nicotinic agonist for the study of CNS nAChRs. Support: NINDS Grant NS 25296; NIEHS Grants ES05730 and T32-ES07263.

*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

**POSTER SESSION 7:
(P7.1-P7.16)**

PRESYNAPTIC MECHANISMS

P7.1

A VESICULAR ACETYLCHOLINE TRANSPORTER AND CHOLINE ACETYLTRANSFERASE ARE EXPRESSED FROM THE SAME HUMAN GENE LOCUS Jeffrey D. Erickson, Hélène Varoqui¹ & Lee E. Eiden, Section on Molecular Neuroscience, Laboratory of Cell Biology, NIMH, NIH, Bethesda, MD, USA; William Modi, Frederick Cancer Research and Development Facility, National Cancer Institute, NIH, Frederick MD 20797; Tom L. Bonner & Ted Usdin, Laboratory of Cell Biology, NIMH, NIH, Bethesda, MD, USA

A human cDNA and cognate gene encoding human VACHT (vesicular acetylcholine transporter) have been cloned and sequenced. Human VACHT, rat VACHT, and other putative VACHTs sequenced to date from *C. elegans* and *Torpedo*, possess a high degree of sequence similarity to the biogenic amine vesicular transporters VMAT1 and VMAT2, but also possess key invariant residues within the transmembrane domains that are conserved among all VACHTs but absent from VMATs, suggesting their importance in imparting specificity of ACh transport to VACHT.

The gene encoding human VACHT is contained on chromosome 10q11.2 within the first intron of the ChAT gene. This arrangement is similar to that observed at the nematode *cha-1* gene locus (A. Alfonso, K. Grundahl, J. R. McManus, J. M. Asbury & J. Rand, J. Mol. Biol., in press, 1994). Both the VACHT and ChAT genes have apparently undergone significant divergence in intron number while retaining the contiguity of the VACHT and ChAT genes at the same locus. ChAT is responsible for the synthesis of acetylcholine, while VACHT is responsible for its sequestration into the vesicular compartment from which its regulated release by exocytosis allows cholinergic neurotransmission to occur. The transcription of both these genes from the same genetic locus provides a mechanism for their co-regulated expression throughout the mammalian cholinergic nervous system, and a unique example of an 'operon-like' organization of a set of genes involved in specification of a neuronal phenotype in the human genome.

¹On leave from Département de Neurochimie, Laboratoire de Neurobiologie Cellulaire, CNRS, 91190 Gif-sur-Yvette, CEDEX, France

P7.2

CLONING OF THE TRANSPORTER PROTEIN (VACHT) THAT MEDIATES ACETYLCHOLINE ACCUMULATION INTO SYNAPTIC VESICLES Jeffrey D. Erickson & Lee E. Eiden, Sec. Mol. Neurosci., Lab. Cell Biol., NIMH, NIH, Bethesda, MD, USA; Hélène Varoqui & Marie-Francoise Diebler Dépt. Neurochim. Lab. Neurobiol. Cell., CNRS, 91190 Gif-sur-Yvette, France; Martin Schäfer & Eberhard Weihe Dept. Anatomy, Johannes-Gutenberg Universität, Mainz, FRG; James Rand, Prog. Mol. Cell Biol., Oklahoma Med. Res. Found., Oklahoma City, OK, USA; Tom L. Bonner & Ted B. Usdin, Lab. Cell Biol., NIMH, NIH, Bethesda, MD, USA

A rat cDNA homologous to the nematode UNC-17 gene product (1) and the *Torpedo* vesamicol binding protein (2) has been cloned and sequenced from a pheochromocytoma (PC-12) cDNA library. Consistent with the proposed function of UNC-17 and the *Torpedo* proteins as vesicular acetylcholine transporters, the rat homolog expresses a binding site for the vesicular ACh uptake inhibitor vesamicol when expressed in primate fibroblasts. To demonstrate that this protein is indeed the vesicular acetylcholine transporter, the ability of monkey CV-1 cells expressing the rat cDNA to accumulate ³H-ACh was examined. These nonneuronal cells possess an acidic intracellular compartment with an associated vacuolar ATPase, but no known neurotransmitter transporters, and have been previously been used to demonstrate functional reconstitution of serotonin and catecholamine transport by the vesicular monoamine transporter (VMAT) (3). ACh accumulation occurs in CV-1 cells expressing the rat PC-12 cell cDNA, but not in mock-transfected controls, is inhibited by vesamicol, and is driven by a pH gradient. This protein has therefore been named VACHT (vesicular acetylcholine transporter).

In situ hybridization histochemistry for VACHT mRNA demonstrates its neuronal expression throughout the cholinergic nervous system both centrally and peripherally. This suggests that, unlike biogenic amine accumulation by VMATs, which exist in two differentially expressed isoforms, a single VACHT can account for ACh vesicular accumulation in cholinergic neurons.

References

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2. H. Varoqui, M.-F. Diebler, F.-M. Meunier et al., *FEBS Lett.* **342**, 97, 1994
3. J. Erickson, L. Eiden, B. Hoffman, *PNAS* **89**, 10993, 1992

P7.3 EFFECTS OF TRIMETHYL-TIN ON CHOLINERGIC NEUROTRANSMISSION Aas, P., Eriksen, S., Kolderup, J.* and Fonnum, F. Norwegian Defence Research Establishment, Division for Environmental Toxicology, P O Box 25, N-2007 Kjeller, Norway. *University of Oslo, Department of Biology, Norway.

The effects of trimethyl-tin (TMT) on the release of acetylcholine (ACh) from parasympathetic nerves have been examined by using guinea-pig trachea as a model. Pre-junctionally, TMT (3 mM) significantly enhanced in a reversible manner the high potassium (75 mM) evoked release of [³H]-ACh. The effect can not be due to stimulation of a pool of ACh which is dependent on extraneuronal calcium for release from cholinergic nerve terminals, since the effect of TMT was not inhibited by low calcium (0.1 mM) or by calcium channel blockers (verapamil, 0.1 mM, flunarizine, 0.1 mM and ω -conotoxin GVIA, 0.2 μ M). The present results also demonstrate that TMT induce emptying of a non-vesicular, probably a cytoplasmic storage pool of ACh, since AH5183 (20 μ M), an inhibitor of the translocation of ACh into synaptic vesicles, had no inhibitory effects on the release of [³H]-ACh evoked by TMT (3 mM). TMT also reduced the neuronal uptake of [³H]-choline and this was probably due to the depolarizing effect of TMT on the cholinergic nerve terminals. Post-junctionally, TMT had no effect by itself or on the carbachol induced smooth muscle contraction, indicating that TMT did not have a general depolarizing effect on smooth muscle cells or an effect on muscarinic receptors. Furthermore, the reduced electrical field induced contraction and the subsequent increase in the basal smooth muscle tension that was observed by addition of TMT was activity-dependent, and was most probably due to emptying of a nervous non-vesicular storage pool of ACh, due to depolarization induced by TMT, as was observed during patch-clamping of GH₄ neuronal cells.

P7.4 ADENOSINE ANTAGONIZES ACETYLCHOLINE RELEASE EVOKED BY ACTION POTENTIALS IN THE ABSENCE OF CALCIUM ENTRY IN FROG MOTOR NERVE Watanabe, M., Silinsky, E.M., Redman, R.S., Oiu, R., and MacDonald, R.C. Depts. of Mol. Pharmacol. and Biolog. Chem., Northwestern U., Chicago, IL 60611 and Evanston, IL 60208.

While the essential need for Ca entry through Ca channels is indisputable for normal secretion, the importance of voltage-sensitive Ca binding proteins as mediators of secretion in vertebrate nerve endings has been a subject of considerable controversy. The target site for adenosine, which has been implicated as a presynaptic mediator of neuromuscular depression, is likewise controversial. We decided to test the possibilities that: i) a voltage-sensitive Ca binding protein promotes phasic acetylcholine (ACh) release and ii) that the action of adenosine might be to impair the functioning of Ca binding proteins associated with the secretory apparatus. We delivered Ca via Ca-containing lipid vesicles (Ca liposomes) to motor nerve terminals in frog cutaneous pectoris muscle under conditions in which Ca entry cannot occur. Neurally-evoked ACh release (recorded as multiquantal end-plate potentials) was generated by Ca liposomes suspended in solutions with no added Ca and containing either a) Mg (1-3 mM), b) Mg (1-3 mM) + Co(1 mM) or c) Mg (1 mM) + EGTA (1 mM). Adenosine (10-100 μ M) inhibited such ACh release generated by nerve impulses in the absence of Ca entry. Both electrophysiological controls and morphological studies using confocal laser microscopy suggest that the liposomes are interacting with the nerve terminal membrane and delivering their entrapped contents to the cytoplasmic milieu without leakage to the exterior of the cell. We also studied the quantitative relationship between adenosine and intracellular Sr (delivered in liposomes) as an activator of the process of evoked ACh release. The results suggest that action potentials in vertebrate motor nerve endings can promote the synchronous, physiologically-functional form of ACh release in the absence of Ca entry provided the cytoplasmic Ca is elevated by Ca liposomes. Such evoked ACh release in the absence of Ca entry is antagonized by adenosine. (Supported by NIH Grants NS12782 and NS 30795.)

P7.5

NIFLUMIC ACID INCREASES POTASSIUM CURRENTS IN FROG MOTOR NERVE TERMINALS AND INHIBITS EVOKED TRANSMITTER RELEASE. C. Solsona, F. Miralles, and J. Marsal. U. Barcelona. Laboratory of Cellular and Molecular Neurobiology. Dept Cell Biology. Medical School. 143 Casanova St. 08036 Barcelona. Spain.

The actions of the nonsteroidal antiinflammatory drug niflumic acid (NA) were studied on frog isolated neuromuscular preparations by conventional electrophysiological techniques. Niflumic acid (100-500 μM) reduced the evoked endplate potentials by 50 to 70%. The increase of extracellular calcium concentration partially reversed the depression of evoked quantal release. NA (100 μM) had only a little effect upon amplitude of miniature endplate potentials (3-20% of reduction). At this concentration NA decreased the frequency of mepps while at 500 μM produced an enormous increase in the rate of the spontaneous discharge. Presynaptic currents recorded at the perineurium changes dramatically when NA (0.1 to 1 mM) was applied to the preparation bath. The deflection associated with presynaptic potassium currents is increased and its kinetics modified. This effect is dose-dependent and partially reversible upon washout. This effect was also recorded when calcium was substituted equimolarly with magnesium. NA increased the amplitude of calcium-activated potassium current and also suppressed the repetitive activity caused by 3,4-diaminopyridine. We suggest that NA reduced the evoked quantal release likely through two mechanisms: activation of presynaptic potassium current and inhibition of prostaglandins synthesis. The effect upon potassium current would shorten the depolarizing phase of the presynaptic action potential and would then reduce the entry of calcium after each impulse. This work is supported by grants from DGICYT, Spanish Government to C.S. and J.M and Fundació August Pi i Sunyer. We are grateful to Prof Dempster (Strathclyde U, Scotland) for the gift of WCP program.

P7.6

QUANTAL NEUROTRANSMITTER RELEASE THROUGH VESICULAR FUSION PORES: MONTE CARLO SIMULATIONS. Stiles, J.R.¹, Bartol, T.M.², Salpeter, M.M.¹, and Salpeter, E.E.³ ¹Section of Neurobiology & Behavior, Cornell University, Ithaca NY; ²Computational Neurobiology Laboratory, Salk Institute, La Jolla, CA; ³Departments of Physics & Astronomy, Cornell University, Ithaca NY.

From studies on mast cell exocytosis (Almers & Tse, 1990, Neuron 4:813-818; Monck & Fernandez, 1994, Neuron 12:707-716), it has been suggested that vesicular neurotransmitter release may occur through a pore with molecular dimensions (i.e. radius ~ 1 nm), and with an e -fold time of 100-300 μs even at synapses such as the neuromuscular junction. However, the 20-80% rise time (t_r) of a miniature endplate current (mEPC) is on the order of 100 μs , and provides a means to rule out vesicular release models which are too slow for such fast synapses. To explore the physiologically plausible range of pore sizes and opening rates, we performed Monte Carlo computer simulations where a pre-synaptic vesicle (radius R) and cylindrical pore (radius r , height h , opening rate dr/dt and dh/dt) were represented explicitly as part of the model synaptic design. The movement of individual neurotransmitter (acetylcholine, ACh) molecules was modeled by means of a 3D random walk. We first determined how the e -fold time for ACh release (τ) depends on R , r , h , and the pore's opening rate, as well as on the apparent diffusion coefficient (D_v) for ACh in the vesicle and pore. For such simulations it was necessary to use a time step in the sub-nanosecond range. We then combined simulation of vesicular release with that of mEPC generation to examine the effect of τ on mEPC t_r and amplitude (A_c). We found that ACh release through a pore with $r \sim 1$ nm gave τ values of sufficient length to significantly reduce A_c and prolong t_r . This was true even when pore opening was instantaneous and D_v was as large as for free diffusion. On the other hand, larger pores with r still less than the vesicle radius (R), and within the range of sizes measured from "omega figures" in electron micrographs (Torri-Tarelli et al., 1985, J. Cell Biol. 101:1386-1399), gave τ values short enough that the efficiency of mEPC generation was unaffected. We propose that a reasonable working model includes diffusional ACh release through a pore which opens at about 10 nm per 100 μs . We conclude that mast cells, in which extremely rapid release of vesicle contents is not required, may not be an adequate model system for neurotransmitter release at fast synapses.

Funded by F32NS09126 (JRS), NS09315 (MMS), and the Howard Hughes Medical Institute (TMB). Certain simulations were conducted at the Cornell National Supercomputer Facility, Ithaca, NY.

P7.7

STABILITY OF PROTEIN COMPLEXES IN TORPEDO CHOLINERGIC SYNAPSES IS AFFECTED BY CALCIUM LEVELS Michal Lintal, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University, Jerusalem, 91904, Israel

Protein complexes at the nerve terminal are crucial for accurate and timely neurotransmitter release. Interference with the stability of such complexes (e.g. by tetanus and botulinum toxins) results in blockage of release. Here we present a biochemical study on *Torpedo* synapses where the stability of protein complexes is shown to be modulated by calcium levels.

VAT-1 is a major 41 kDa protein of the synaptic vesicle membrane. Its hydrodynamic characterization suggests that it is composed of 4 subunits which form a protein complex of 170 kDa. The binding of these subunits is partially disrupted by chelating calcium ions. Recombinant VAT-1 is, in itself, a low-affinity calcium binding protein. This result suggests that structural changes by calcium concentrations might serve to modulate synapse activity.

The effect of calcium on the stability of protein complexes is also evidenced by our study on *Torpedo* syntaxin. Syntaxin is a key component in a protein complex which was implicated in vesicular targeting as well as in docking and priming of synaptic vesicles. Syntaxin in *Torpedo* is highly enriched in synaptosomes from *Torpedo* electric organ as was detected by antibodies raised against mammalian syntaxin. Following gel electrophoresis, *Torpedo* syntaxin was resolved into 2-3 discrete bands of 35-36 kDa which correspond to different modified forms of a single protein. Changes in calcium concentration during synaptosomes solubilization altered the number of distinct syntaxin variants as well as their relative quantities.

Torpedo syntaxin was found primarily in two high molecular weight protein complexes of 8S and 4-5 S. The distribution of syntaxin variants in these complexes is also calcium dependent. Specifically, we observed that the distribution of the unmodified variant is calcium independent, while one of the modified variants was detected in the 8S protein complex only at the presence of calcium. Nevertheless, the syntaxin-VAMP/synaptobrevin as well as syntaxin n-secl interaction was shown to be calcium independent. Our findings demonstrate how calcium levels modulate *Torpedo* syntaxin and consequently affect its interaction with other key components of the docking-release apparatus.

This work was supported by the Ministry for science and arts and by the Israeli Academy of Science.

P7.8

SOMATOSTATIN INHIBITS ACETYLCHOLINE RELEASE BY MODULATING CALCIUM CHANNELS VIA A cGMP- AND NITRIC OXIDE-DEPENDENT PATHWAY IN CILIARY GANGLION CELLS. D.B. Gray, S.D. Meriney, and G.R. Pilar. Department of Physiology and Neurobiology, University of Connecticut, Storrs, CT.

In avian ciliary ganglion (CG) terminals, somatostatin (SOM) is a potent inhibitor of acetylcholine (ACh) release evoked by potassium or electrical stimulation. In intact terminals, this inhibition requires generation of nitric oxide (NO) and activation of cGMP-dependent protein kinase (PKG) (Gray et al., 1993) Soc. Neurosci Abstr, 19:11777. In this study, labeled ACh release was measured from embryonic day 14 chick ciliary ganglion cells acutely isolated and plated on polyornithine coated wells. In these cells, ACh release evoked by a 5 min incubation in 55 mM KCl Tyrodes is calcium dependent, and is completely blocked by 500 nM SOM. As in intact terminals, SOM-induced inhibition of evoked release can be significantly reversed by preincubation of cells with a specific PKG inhibitor, 100uM 8-para-chlorophenylthioguanosine -3',5'-cyclic monophosphorothioate, Rp isomer (Rp-8-pCPT cGMPs), or with a specific inhibitor of NO synthase: l-nitroarginine monomethyl ester (l-NAME). Conversely incubation of CG cells with either Sp-8-pCPT cGMPs, a PKG agonist, or sodium nitroprusside, an NO donor blocks potassium-evoked ACh release mimicking the application of SOM. In a companion abstract (this meeting) Metzger et al., report direct effects of Rp-8-br-pCPT cGMPs and l-NAME on SOM inhibition of voltage-dependent calcium currents measured via the perforated patch technique in acutely isolated CG cells. These antagonists lead to an immediate relaxation of current inhibition and desensitization of the SOM effect as often seen in the whole cell recording method. These results indicate that activation of the SOM receptor leads to modulation of neurotransmitter release by inhibition of calcium channels via a soluble intracellular pathway utilizing both NO and PKG. Supported by NSF IBN# 9213204, Univ. of Pittsburgh Small Grants Program, Winters Foundation Award, Simmons College Fund for Research, and NIH NS 32345.

P7.9

SOMATOSTATIN-MEDIATED INHIBITION OF Ca²⁺ CURRENT MODULATED BY cGMP-DEPENDENT KINASE AND NITRIC OXIDE.

Metzger, R.R.¹, Crumling, M.A.¹, Gray D.B.², Pilar, G.R.³ & S.D. Meriney¹.
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Direct measurement of ACh release from intact synapses of ciliary ganglion neurons has implicated nitric oxide and cGMP-dependent protein kinase (PKG) in a cascade that couples somatostatin (SOM) receptors to inhibition of ACh release (Gray et al., Soc. Neurosci. Abst. 19:1177). Here, we report the use of perforated patch recordings from acutely dissociated ST 40 ciliary ganglion neurons to test the hypothesis that these effects are mediated through inhibition of calcium current. In these cells, SOM inhibited Ca²⁺ current in a pertussis toxin-sensitive manner in both whole cell and perforated patch recordings. In perforated patch recordings, SOM effects were robust and persistent. In contrast, SOM effects rapidly desensitized in whole cell recordings suggesting the loss of a soluble component. This desensitization could be reproduced in perforated patch recordings when either PKG was inhibited by 100 μM Rp-8-pCPT-cGMPs or nitric oxide synthase was inhibited by 10 mM L-NAME. Activation of PKG by Sp-8-Br-cGMPs or dibutyryl-cGMP mimicked the effect of SOM on Ca²⁺ current. In a companion abstract (this meeting), Gray et al. have shown that ACh release from these acutely dissociated somata was blocked by SOM, and that this inhibition could be reversed by either Rp-8-pCPT-cGMPs or L-NAME. Therefore, ciliary ganglion neuronal somata are functional models for SOM-mediated modulation of ACh release. Our study reveals a SOM receptor-triggered signal transduction cascade that includes both membrane-delimited and soluble messengers that together cause a sustained inhibition of neuronal Ca²⁺ current leading to a decrease in transmitter release. Supported by the University of Pittsburgh Small Grants Program, a Winters Foundation Award, NIH NS 32345 and NSF IBN 9213214.

P7.10

TRANSMITTER PACKET CLASSES AND SIZES ARE DETERMINED BY A DYNAMICAL PROCESS AT THE MOMENT OF RELEASE. Kriebel, M.E. and Bridy, D.J.
 Dept. of Physiology., SUNY Health Science Center, Syracuse, New York, 13210, USA.

Tenets of the quantal-vesicular hypothesis are one class of immutable packets prepackaged in synaptic vesicles; and, an exocytotic release mechanism governed by random rules. Spontaneous (MEPPs) and evoked packet release are from the same pool and the size of the evoked endplate potential (EPP) depends on the number of available quanta and the probability of release. During early stages of synaptogenesis most MEPPs belong to a skew class which is 1/7th the size of the MEPPs first studied by Fatt and Katz (termed bell-MEPPs because they form a bell-shaped distribution). During maturation, the skew-class decreases to 4% in the adult frog and mouse neuromuscular junction, but later increases to 50% in the old mouse. The two classes have different Arrhenius constants, physiological and pharmacological properties. The ratio of skew- to bell-MEPPs is increased with calcium ions, lanthanum ions, potassium ion depolarization, ionophores, beta-bungarotoxin, botulinum toxin, nerve stimulation, hypertonic saline, black widow spider venom, and heat treatment. Most of these treatments also increase the number of "giant-MEPPs" which belong to the skew-class and also change the size of the bell-MEPP. Both skew- and bell-MEPP distributions show multiple peaks which demonstrate a sub-unit composition. The mode of the skew class is stable except under extremely high rates of release. There are too many MEPPs with steps the size of the skew-MEPP mode (sub-MEPP) to occur by chance. Slow MEPPs and giant skew-MEPPs also show a substructure. We report that the distribution of intervals between MEPPs is frequency dependent. Small patches of endings (2 micron dia) from the Torpedo electrocyte were focally recorded. At low frequencies, a log-log plot of number vs interval size fits a power law distribution whereas at high frequencies the distribution is flat for the first 5 interval classes which shows preferred frequencies. In a second series of experiments we selected 6 micron diameter frog muscle fibers which showed only 0.03 MEPP/sec. MEPP frequency was increase 50 fold with ethanol. Number-interval plots showed peaks every 4.25 bins (400 Hz). Periodograms also showed a 400 Hz peak with several secondary peaks. These data indicate that the release process can change dynamics. We propose that a release channel could meter transmitter in subunit amounts to generate the substructure of the two classes. (supported by NIH NS25683 and NSF 19694)

P7.11

SECRETION INDUCED BY NICOTINE DOES NOT REQUIRE Na^+ CHANNEL ACTIVATION IN PORCINE ADRENAL CHROMAFFIN CELLS. Forsberg, E.J. and Li, Q. Dept. Physiology, Univ. of Wisconsin, Madison, WI, USA.

Secretion induced by nicotinic agonists in adrenal chromaffin cells is thought to depend to a large extent on the depolarization produced by the opening of nicotinic receptor channels. Membrane depolarization is thought to open voltage-gated Na^+ channels, leading to the generation of action potentials and the subsequent activation of voltage-gated Ca^{2+} channels. This hypothesis is supported by our observation that removal of extracellular Na^+ blocks secretion and increases in $[\text{Ca}^{2+}]_{\text{in}}$ produced by nicotine but has no effect on responses to elevated extracellular K^+ . However, we have found that tetrodotoxin, which abolishes fast Na^+ currents produced by depolarization of voltage-clamped chromaffin cells, has no effect on either secretion or increases in $[\text{Ca}^{2+}]_{\text{in}}$ produced by nicotine. The effect of removing Na^+ on nicotine-induced secretion and increases in $[\text{Ca}^{2+}]_{\text{in}}$ can be reversed by adding excess extracellular Ca^{2+} (20 mM). Under conditions of high extracellular Ca^{2+} and no Na^+ , the dihydropyridine Ca^{2+} channel blocker, nimodipine (2 μM), abolishes both nicotine- and elevated extracellular K^+ -induced secretion. These results suggest that voltage-gated Ca^{2+} channels in porcine chromaffin cells can be activated by depolarizations produced by Ca^{2+} entry through the nicotinic receptor channel. *This work was supported by the Wisconsin Alumni Research Foundation.*

P7.12

THE ROLE OF PROTEIN KINASE C IN FREQUENCY-DEPENDENT FACILITATION IN THE ISOLATED CHICK OESOPHAGUS. Searl, T.J. Department of Pharmacology, University of Oxford, Oxford, U.K.

Very little is known about the mechanisms regulating acetylcholine (ACh) release at muscarinic neuroeffector junctions. There are several reasons for this, the most important being the complexity of the processes linking muscarinic receptors to their eventual electrical responses. The smooth muscle of the chick oesophagus is a preparation which exhibits fast muscarinic responses. During short trains of electrical stimuli excitatory junction potentials (EJPs) show marked frequency-dependent facilitation. In contrast, pulses of ionophoretically applied ACh show no similar facilitation, suggesting that nerve-evoked frequency-dependent facilitation is prejunctional in origin. The protein kinase C inhibitor Ro-31,8220 (10 μM) abolished frequency-dependent facilitation (see figure).



These findings suggest that protein kinase C plays a fundamental role in regulating acetylcholine release in the chick oesophagus.

P7.13

LOCALIZATION OF Na/Ca EXCHANGER AND ER Ca-ATPase IN NEURONS AND ASTROCYTES. Juhászová, M., Golovina, V., Tribe, R.M., and Blaustein, M.P. Dept. Physiol., U. MD Med. Sch., Baltimore, MD 21201.

Na/Ca exchange, driven by a transmembrane Na⁺ gradient, helps to regulate both [Ca²⁺]_{cyt} and the intracellular stores of Ca²⁺ in neurons and glia. Immunoblotting was used to determine the presence and prevalence of the Na/Ca exchanger in synaptic plasma membranes (SPM) and astrocytes. Polyclonal antibodies raised against dog cardiac sarcolemmal exchanger specifically crossreacted with 120 and 70 kDa bands in both SPM and astrocytes. More Na/Ca exchanger was detected in SPM than in astrocytes. The Na/Ca exchanger activity appears to be closely coupled to Ca²⁺ availability from intracellular stores. This raises questions about whether this functional coupling of transport systems has a structural/spatial correlate. The distribution of relevant membrane ion transporters was studied with digital imaging microscopy. To identify the endoplasmic reticulum ER and mitochondria we used the fluorochrome, DiOC₆(3). Mitochondria were clearly stained by 0.05 μg/ml DiOC₆(3), whereas 0.5 μg/ml destroyed the mitochondria and revealed the full extent of the ER reticular network in both neurons and astrocytes. The 3-D reticular ER pattern was identified in deconvolved, restored images. Immunofluorescent labeling was employed to localize and co-localize, plasmalemmal Na/Ca exchanger and ER Ca-ATPase. The ER Ca-pump was visualized with antibodies raised against the SERCA-2b Ca-ATPase isoform. Ca-ATPase molecules were distributed all along the ER [identified with DiOC₆(3)] of both neurons and astrocytes. Immunofluorescent labeling of the Na/Ca exchanger was distributed in a reticular pattern over the surface of cultured rat astrocytes. This suggests that the exchanger in astrocytes may be localized to plasmalemmal domains that overlie subplasmalemmal (junctional) ER. This spatial proximity of the Na/Ca exchanger and some ER Ca²⁺ pumps in astrocytes provides a structural basis for the functional coupling mentioned above. The distribution of the Na/Ca exchanger in neurons is quite different. Although punctate labeling occurs on neurites and soma (where it may overlie junctional ER), the label is concentrated at presynaptic terminals (Luther et al., *J. Neurosci.* 12: 4898-4904, 1992), where it apparently plays a special role in Ca²⁺ extrusion and regulation of neurotransmitter release.

P7.14

COMPARISON OF THE EFFECTS OF VARIOUS nNACHR AGONISTS ON NEUROTRANSMITTER RELEASE IN THE HIPPOCAMPUS, THALAMUS AND STRIATUM OF THE RAT. Sacaan, A.I., Dunlop, J., Lloyd, G.K. SIBIA, 505 Coast Blvd. South, La Jolla, California 92037.

The effects of the neuronal nicotinic acetylcholine receptor (nNACHR) agonists Nicotine (Nic), Cytisine (Cyt) and dimethylpiperazinium iodide (DMPP) on ³H-DA release from rat striatal slices and ³H-NE release from rat hippocampal and thalamic slices are summarized below.

Compound	Striatum	EC ₅₀ (μM) n = 3-7	
		Hippocampus	Thalamus
Nic	1.28	39.3	16.6
Cyt	0.06	13.1	17.1
DMPP	0.83	04.8	14.0

The different rank order of potency for the three agonists in the various tissues studied suggests that distinct nNACHR subtypes modulate the release of neurotransmitters in different tissues. The nicotinic agonist-induced ³H-DA release was TTX-insensitive confirming earlier reports that nNACHR subtypes involved in releasing DA are located on the DA-ergic nerve terminal. In contrast, as the effects of all agonists on NE release were TTX-sensitive, the nNACHR subtypes involved in modulating NE release in either the hippocampus or the thalamus are not located directly on the NE nerve terminals.

In addition, all agonists were equiefficacious in stimulating ³H-NE release in the hippocampus and the thalamus. In the striatum, cytisine acted as a partial agonist producing a maximum of 53% of ³H-DA release as compared to that of Nic. The responses to agonists varied in antagonist sensitivity. In the hippocampus, ³H-NE release induced by all three agonists was significantly reduced by Mec (1 μM) and d-TC (10 μM). In contrast, the effects of the three agonists in the thalamus were Mec but not d-TC sensitive. Finally in the striatum, Mec significantly inhibited the effects of Nic, Cyt and DMPP while d-TC only inhibited the effect of Nic.

These results suggest that different nNACHR subtypes are involved in modulating neurotransmitter release in different brain areas.

P7.15 PUTATIVE VESICULAR NUCLEOTIDE TRANSPORTER IS GAPDH. Volkandt, W., Schläfer, M., and Zimmermann, H. AK Neurochemie, Zoologisches Institut, Biozentrum der J.W. Goethe-Universität, Frankfurt am Main, Germany.

Synaptic vesicles isolated from electric ray electric organ have previously be shown to contain a 34 kDa protein that binds azido-ATP, azido-AMP, and N-ethyl-maleimide. The protein was found to share similarities with the mitochondrial ADP/ATP carrier and assumed to represent the synaptic vesicle nucleotide transporter. Synaptic vesicles were purified by sucrose density gradient centrifugation and subsequent chromatography on Sephacryl S-1000 from both *Torpedo* electric organ and bovine brain cerebral cortex. They contain ATP-binding proteins of 35 kDa and 34 kDa, respectively. ATP binding is inhibited by AMP. Both proteins are highly enriched after column chromatography of vesicle proteins on AMP-Sepharose. Antibodies were obtained against both proteins. Antibodies against the bovine brain synaptic vesicle protein of 34 kDa specifically bind to the 35 kDa protein of *Torpedo* vesicles. An NH₂-terminal sequence obtained against the 34 kDa protein of bovine brain synaptic vesicles identifies it as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The previously observed molecular characteristics of the putative vesicular nucleotide transporter in *Torpedo* fit to those of GAPDH. We therefore suggest that the protein previously identified as putative nucleotide transporter is in fact vesicle-associated glyceraldehyde-3-phosphate dehydrogenase.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 169/A 10) and a personal travel grant for W.V. from the NATO.

P7.16 CA1 PYRAMIDAL CELL CONTROL OF GABAERGIC INPUT IS ENHANCED BY MUSCARINIC RECEPTOR ACTIVATION. Pitler, T.A. and Alger, B.E. Dept. Physiol., University of Maryland Sch. Med., Baltimore, MD, USA.

Interactions among the effects of different neurotransmitter systems form a complex and little-understood topic. In hippocampus both muscarinic and GABAergic systems play important roles in epilepsy, theta rhythm and LTP. We have begun to study muscarinic-GABAergic interactions in the CA1 region of submerged rat hippocampal slices with whole-cell recordings using the "blind" impalement technique.

Application of muscarinic agonists or stimulation of cholinergic fibers in the slice greatly increases spontaneous GABA IPSCs in pyramidal cells via activation of receptors on the interneurons (Pitler and Alger, 1992, J. Physiol., 450:127-142). We have also described another GABA-related response, depolarization-induced suppression of inhibition (DSI) in which depolarization of a pyramidal cell by action potentials or voltage pulses (to 0 mV, 1 sec) causes a dramatic, Ca²⁺-dependent, reduction in GABAergic IPSCs for ≥ 20 sec (Pitler and Alger, 1992, J. Neurosci., 12:4122-4132). TTX-resistant miniature IPSC amplitude is not reduced and there was little change in mIPSC frequency; iontophoretic GABA responses are unaffected (Pitler and Alger, 1994, Soc. Neurosci. Abstr., in press). Hence, although DSI requires pyramidal cell activation for its induction, it is expressed by a reduction in GABA output from the interneuron. A retrograde signal may be involved.

We now report that spontaneous IPSCs induced by muscarinic receptors are unusually susceptible to DSI. These IPSCs, evoked by bath application of carbachol, 25 μ M, or stimulation of cholinergic fibers in *s. oriens* (50 Hz, 0.5 sec), are usually larger than baseline spontaneous IPSCs and can be dramatically reduced during DSI, even when the baseline IPSCs are unaffected. This is most dramatically seen in single-cell recordings in which DSI occurs only during the period of evoked ACh release. Identical protocols applied at other times are ineffective. The reasons for this muscarinic enhancement are currently under investigation. This work is supported by NIH NS30219 (BEA) and a Bressler Foundation Award (TAP).

*Third International Symposium
The Cholinergic Synapse:
Structure, Function and Regulation*



UNIVERSITY OF MARYLAND
AT BALTIMORE

NOTES

**POSTER SESSION 8:
(P8.1-P8.22)**

PHARMACOLOGY OF NICOTINIC RECEPTORS

P8.1

HETEROGENEITY OF PRESYNAPTIC NICOTINIC ACETYLCHOLINE RECEPTORS. *Mike, A., #Sershen, H., *Balla, A., #Lajtha, A. and *Vizi, E.S., *Dept. of Pharmacology, Institute of Experimental Medicine, Hung. Acad. Sci., Budapest, Hungary. #Center for Neurochemistry, Nathan Kline Institute, Orangeburg, New York, USA

Our study attempted to characterize the subunit composition of nicotinic acetylcholine receptors (nAChRs) participating in pre- and postsynaptic mechanisms of neural transmission, and to study the role of voltage-sensitive calcium channels in the nAChR-mediated events. For these experiments we assayed the effects of a number of nicotinic agonists, including the potent novel analgetic epibatidine, using several preparations. In guinea pig ileum-myenteric plexus and vas deferens and rat hippocampal slices, effects of the compounds on norepinephrine release were studied by a superfusion technique, while effects on cultured rat hippocampal neurons were studied by whole-cell voltage clamp. The rank order of potency of agonists suggests that the predominant type of nAChRs present in the presynaptic noradrenergic nerve terminals of the rat vas deferens and hippocampus, as well as those present on other nerve terminals are composed of $\alpha 3\beta 2$ subunits, whereas in the guinea-pig myenteric plexus the somatodendritic nAChRs are composed of $\alpha 4\beta 2$ subunits.

P8.2

HETEROGENEITY IN FUNCTIONAL NICOTINIC RECEPTORS AND SINGLE CHANNELS IN CHICK BRAIN SLICES. Chiappinelli, V.A. and Weaver, W.R. Dept. of Pharmacol. and Physiol. Science, Saint Louis Univ. Sch. Med., St. Louis MO, USA.

We have used intracellular and patch-clamp electrophysiology to examine the functional properties of native neuronal nicotinic acetylcholine receptors (nAChRs) in neurons within embryonic chick brain slices (17-21 days of incubation). In the lateral spiriform nucleus (SPL), nAChRs with a high affinity for nicotine and insensitivity to α - and kappa-bungarotoxin are found postsynaptically on nearly all SPL neurons (Sorenson and Chiappinelli, *Neuron* 5, 1990;307-315). Using a wide range of concentrations of both agonist (3 μ M - 3 mM carbachol in the presence of 1 μ M atropine) and antagonists (0-500 μ M) in superfusion studies, we find that the nicotinic antagonist dihydro- β -erythroidine exhibits a narrow range of K_i values against these receptors in individual SPL neurons (K_i = 0.09-0.16 μ M). In marked contrast, K_i 's determined for trimethaphan using identical procedures yielded a wide range of potencies for this antagonist (K_i = 4-66 μ M). The results demonstrate that multiple subtypes of high affinity nicotine receptors are present on SPL neurons, and that trimethaphan distinguishes between nAChR subtypes in the central nervous system. Since SPL neurons are known to express the $\alpha 2$, $\alpha 4$, $\alpha 5$ and $\beta 2$ nicotinic receptor subunits, there is molecular biological evidence to account for this functional heterogeneity.

We have recently begun to analyze single nicotinic receptor channels in cell-attached patches on SPL neurons within chick brain slices. With 1-30 μ M acetylcholine in the patch pipet, we detect single channels exhibiting properties expected for nAChR channels, including reversal potentials from -30 to +30 mV, marked rectification at depolarized potentials, and slope conductances averaging 37 pS (n=17). These channels are not observed when acetylcholine is not present in the pipet. Evidence for heterogeneity in these single nAChR channels includes channels with different slope conductances (range: 20-60 pS) and a range of open times.

Supported by NIH Grant NS17574 to V.A.C.

P8.3

FUNCTIONAL DIVERSITY OF NICOTINIC ACh RECEPTOR-CHANNELS IN PARASYMPATHETIC NEURONS OF RAT INTRACARDIAC GANGLIA.

Nutter, T.J. and Adams, D.J., Department of Molecular & Cellular Pharmacology, University of Miami School of Medicine, Miami, FL USA.

Nicotinic acetylcholine receptor (nAChR)-mediated currents in cultured neurons of rat intracardiac ganglia were examined using patch clamp techniques. The whole-cell, ACh-evoked current-voltage (I-V) relationship exhibited marked inward rectification in symmetric Na⁺ solutions. nAChR sensitivity was examined by brief (10 ms) pressure application of various nicotinic receptor agonists to the voltage-clamped soma. The rank order of agonist potency determined from peak current amplitude evoked by 100 μM concentrations of acetylcholine, cytisine, nicotine, and 1,1-dimethyl-4-phenylpiperazinium (DMPP) varied from cell to cell. Approximately half of the cells responded in the following order of agonist potency: ACh > DMPP > cytisine > nicotine. The order of potency in 40% of the neurons was ACh > cytisine > DMPP > nicotine and 10% exhibited the sequence: cytisine > ACh > nicotine > DMPP. Differences in agonist potency have been reported for neuronal nAChRs composed of different subunit combinations [Luetje & Patrick, 1991, *J. Neurosci.* 11:837-845]. The single channel conductance of the nAChR was examined in excised outside-out membrane patches. The unitary i-V relationship was linear in symmetric Na⁺ solutions, with a mean slope conductance of 24.1 ± 0.8 pS (n=15). ACh-induced single channel currents observed in excised patches exhibited three distinct conductance levels (30.3 ± 0.6 pS, 22.1 ± 0.4 pS and 16.1 ± 0.7 pS). The 22 pS conductance level was observed in all membrane patches exhibiting ACh-activated currents. Fifty percent of the patches exhibited both the 16 pS conductance level (40% of channel openings) and the 22 pS conductance level (~60% of channel openings). In 30% of the excised patches the 30 pS (60% of openings) and 22 pS (40% of openings) conductance levels were observed. All three conductance levels were present in 20% of the neurons examined, with ~30% of the channel openings having a 16 pS conductance, 50% a 22 pS and 20% a 30 pS conductance. Taken together, these results suggest that the differences observed in unitary conductances and agonist sensitivities may reflect the expression of multiple nAChR subunit combinations in parasympathetic neurons of rat intracardiac ganglia. [Supported by NIH HL-35422]

P8.4

FUNCTIONAL PHARMACOLOGY OF THE NOVEL NICOTINIC ACETYLCHOLINE RECEPTOR AGONIST ABT-418 IN PC12 CELLS. Briggs, C.A., Hughes, M.L.,

Monteggia, L.M., Giordano, T., Donnelly-Roberts, D. and Americ, S.P. Neuroscience Research, D-47W Bldg. AP10, Abbott Laboratories, Abbott Park, IL, U.S.A. 60064-3500.

The pharmacologic and physiologic diversity of nicotinic acetylcholine receptors (nAChRs) suggests possibilities for developing novel nicotinic agonists that have reduced adverse effects while retaining apparent positive attributes of (-)nicotine such as cognitive enhancement and anxiety reduction. One example, ABT-418, is demonstrated to activate ionotropic nAChR in PC12 cells as a model for the ganglionic subtypes.

PC12 cells were maintained in Dulbecco's Modified Eagle Medium containing 10% heat-inactivated fetal calf serum and 5% heat-inactivated horse serum. Differentiation of the cells was induced by exposure to nerve growth factor (100 ng/ml) for 4-7 days. The whole-cell patch-clamp technique was used to record ligand-gated currents activated by substances applied at defined concentrations using the U-tube flow-reversal technique. The holding potential was -60 mV. The extracellular solution contained 150 mM NaCl, 2.8 mM KCl, 2.0 mM CaCl₂, 1.0 mM MgCl₂, ≥ 10 mM dextrose and 10 mM Na-HEPES buffer (7.3 pH, 325 mOsm) while the intracellular (recording pipette) solution contained 140 mM KCl, 1.0 mM CaCl₂, 2.0 mM MgCl₂, 11 mM K-EGTA, and 10 mM K-HEPES buffer (7.3 pH, 315 mOsm).

ABT-418 activated inward currents with a potency 4-fold lower than that of (-)nicotine (EC₅₀ = 214 μM and 52 μM, respectively). The efficacy of ABT-418 was not significantly different from (-)nicotine (89%). Responses to 300 μM ABT-418, like those to 100 μM (-)nicotine, were reversibly inhibited 81% by 10 μM mecamylamine, 38% by 10 μM dihydro-β-erythroidine, and 82% by 100 μM dihydro-β-erythroidine. Furthermore, responses to maximal concentrations of ABT-418 (3 mM) and (-)nicotine (1 mM) were not additive. However, the Hill coefficient for ABT-418 (1.18) was smaller than that for (-)nicotine (1.77), and high concentrations of ABT-418 appeared to elicit a more rapidly decaying response than did (-)nicotine. Thus, ABT-418 activated ganglion-like nAChR channels in PC12 cells with a slightly lower potency than (-)nicotine and with only subtle differences in the response characteristics.

P8.5

NICOTINIC RESPONSES IN CULTURED PHEOCHROMOCYTOMA (PC12) CELLS.
 E.F.R. Pereira and Albuquerque, E.X. Dept. Pharmacol. Exp. Ther., Univ. Maryland
 Sch. Med., Baltimore, MD 21201, USA.

Although clonal rat PC12 cells represent an established model for the study of sympathetic ganglionic transmission and express various members of the nicotinic receptor (nAChR) gene family (Henderson *et al.*, *J. Neurosci.*, *in press*, 1994), the characteristics of nicotinic currents evoked in these cells have not been assigned to specific nAChR subtypes. In the present study, we have attempted to characterize the neuronal nAChR that subserves nicotinic currents evoked in cultured PC12 cells by comparing the characteristics of these currents with those of nicotinic currents activated in cultured hippocampal neurons. Application of ACh (3 μ M - 3 mM) *via* a U tube to PC12 cells evoked whole-cell currents whose peak amplitudes increased with the concentration of ACh: the EC₅₀ for ACh was about 75 μ M. Using Mg²⁺-free, Cs⁺-based internal solution, ACh-evoked currents showed a mild inward rectification, resembling type IA currents evoked in cultured hippocampal neurons. In contrast to type IA currents, however, ACh-evoked currents in PC12 cells decayed with a slow time constant. Also, dihydro- β -erythroidine (10 nM), a competitive antagonist of ACh at α 4 β 2 neuronal nAChRs (Alkondon and Albuquerque, *J. Pharmacol. Exp. Ther.*, **265**:1455, 1993), had no effect on ACh (3 mM)-evoked nicotinic currents in PC12 cells, whereas methyllycaconitine (MLA, 1 nM), a competitive antagonist of ACh at α 7-bearing nAChRs (Alkondon and Albuquerque, *J. Pharmacol. Exp. Ther.*, **265**:1455, 1993), inhibited the activation of these currents. A long-lasting effect was observed when MLA was applied to the PC12 cells *via* the U tube and the background perfusion. Although the pharmacological profile of nicotinic currents activated in PC12 cells resembles that of MLA-sensitive, IA currents activated in hippocampal neurons, the slow decay phase and the MLA-induced, long-lasting blockade of nicotinic currents in PC12 cells were different from the characteristics of IA currents. These results suggest that the neuronal nAChRs expressed in PC12 cells are different from the neuronal nAChRs characterized to date.
Support: NIH grants NS 25296 and ES 05730.

P8.6

DIFFERENTIAL EFFECTS OF LEAD ON SUBTYPES OF NEURONAL NICOTINIC RECEPTORS IN NEUROBLASTOMA CELLS AND XENOPUS OOCYTES
 Oortgiesen, M., Zwart, R. Van Kleef, R.G.D.M., and Vijverberg H.P.M.
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Inorganic lead (Pb²⁺) blocks synaptic transmission, which is generally thought to be due to presynaptic block of voltage-dependent calcium channels. Recently, it was found that neuronal type nicotinic acetylcholine receptors (nAChR) are highly sensitive to Pb²⁺. N1E-115 neuroblastoma cells express neuronal nAChRs, which are insensitive to α -bungarotoxin and is blocked nanomolar concentrations of by k-bungarotoxin. Pb²⁺ affects inward currents induced by activation of nAChR in N1E-115 cells in a dual manner. Between 1 nM and 3 μ M Pb²⁺ the ACh-induced inward current is blocked in a concentration-dependent manner (IC₅₀=19 nM, E_{max}=90%). At higher concentrations (10-100 μ M) the blocking effect on the nAChR is reversed, while the kinetics of the inward current are delayed (EC₅₀=21 μ M). Both the block and the reversal of block do not to interfere with receptor desensitization or with ion channel block by ACh itself and appear to be due to a non-competitive interaction.

The *Xenopus* oocyte expression system was used to further examine the dual nature of the effect of Pb²⁺ on specific nAChR subtypes. Effects of Pb²⁺ were investigated in voltage clamped oocytes expressing nAChR after co-injection of α 3 and β 2 or α 3 and β 4 subunit cDNA. At 1-250 μ M, Pb²⁺ causes a 10-1000% increase in the peak amplitude of ACh-induced inward currents mediated by the α 3 β 2 nAChR. Only blocking effects of Pb²⁺ are observed on α 3 β 4 nAChR. The inhibitory potency of lead greatly varies between cells. In 50% of the cells tested (n=36) concentrations \leq 1 μ M Pb²⁺ blocked the ACh-induced inward current by 31-93%. In the other cells even at higher concentrations (up to 100 μ M) Pb²⁺ caused only 0-65% inhibition.

These results show that Pb²⁺ may potentiate as well as block nAChR, depending on the combination of nAChR subunits expressed. In this respect analogy exists between the dual action of Pb²⁺ on native nAChR in neuroblastoma cells and on nAChR expressed in oocytes. The differential distribution subunit mRNA in the central nervous system suggests the presence of distinct types of nAChRs, which may have different physiological function as well as different sensitivities to neurotoxic substances.

P8.7

ONTOGENY OF NICOTINIC RECEPTORS IN HIPPOCAMPAL NEURONS AND THEIR SENSITIVITY TO LEAD. K. Ishihara, M. Alkondon and E.X. Albuquerque.
Dept. Pharmacol. Exp. Ther., Univ. Maryland School of Medicine., Baltimore, MD 21201.

The presence of nicotinic acetylcholine receptors (nAChRs) on rat hippocampal neurons was studied using the whole-cell patch-clamp technique. Hippocampal neurons were obtained from fetal rats and grown in culture for several days, or were obtained by acute dissociation from postnatal rats at different days after birth. Acetylcholine (ACh, 3 mM) evoked a rapidly desensitizing whole-cell current (referred to as type IA) in a large percentage of the neurons tested. In a small population of neurons, ACh could evoke a slowly desensitizing current (referred to as type II). Type IA currents were sensitive to blockade by α -bungarotoxin (10 nM) and by methyllycaconitine (1 nM), and showed strong inward rectification in the presence of intracellular Mg^{2+} . Type II currents were blocked by dihydro- β -erythroidine (100 nM), and showed strong inward rectification even in the absence of Mg^{2+} in the intracellular medium. The peak amplitudes of type IA currents increased as the neurons developed both *in vitro* and *in vivo*. However, the mean peak amplitude of type IA current evoked in acutely dissociated neurons from postnatal rats was much smaller than that elicited in the fetal neurons grown in culture. The mean amplitude of type IA currents was substantially decreased by acute exposure of the hippocampal neurons to Pb^{2+} . While Pb^{2+} inhibited type IA currents with an apparent IC_{50} of 3 μM , about 30 μM was necessary to reduce the peak amplitude of type II currents by 50%. Pb^{2+} did not affect the decay time constants of type IA or type II currents. Pb^{2+} affected neither the frequency nor the kinetics of ACh-evoked, single-channel currents in frog muscle fibers in the cell-attached configuration. The present results suggest that i) functional nAChRs are present on hippocampal neurons that develop *in vivo* and *in vitro*; ii) α -bungarotoxin-sensitive neuronal nAChRs are more sensitive to the inhibitory effect of Pb^{2+} compared to other nAChR subtypes. Support: NINDS Grant NS 25296; NIEHS Grants ES05730 and T32-ES07263.

P8.8

MAPPING DETERMINANTS OF COMPETITIVE ANTAGONIST SENSITIVITY ON NEURONAL NICOTINIC RECEPTOR SUBUNITS. Harvey, S.C., Maddox, F.N. and Luetje, C.W. Department of Molecular and Cellular Pharmacology, University of Miami, Miami, Fl. 33101

Neuronal nicotinic acetylcholine receptors (nAChR) can be formed in *Xenopus* oocytes by injecting various combinations of cRNAs encoding two classes of homologous subunits, α and β . Each subunit combination has distinct pharmacological properties, with both α and β subunits contributing to ligand sensitivity. The $\alpha 3\beta 2$ subunit combination is sensitive to block by 100 nM neuronal bungarotoxin (NBT) ($98.0 \pm 1.9\%$ block) while $\alpha 2\beta 2$ and $\alpha 3\beta 4$ are insensitive. Previous work has shown that the contribution of $\alpha 3$ to the NBT sensitivity of $\alpha 3\beta 2$ can be localized to three distinct sequence segments (84-121, 121-181, 195-215), and that gln198 of $\alpha 3$ (pro in $\alpha 2$) plays a role in determining NBT sensitivity. We have made a series of mutations within these regions of $\alpha 3$, changing residues from what occurs in $\alpha 3$ to what occurs in $\alpha 2$. Changing thr143 of $\alpha 3$, to lys as in $\alpha 2$ (T143K), results in a loss of NBT sensitivity ($7.7 \pm 4.6\%$ block by 100 nM NBT). Amino acid changes in $\alpha 3$ that had no effect on NBT sensitivity include K87I, Q101A, L109H, K111F, K129S, and Y139Q. The $\alpha 4$ subunit forms receptors (with $\beta 2$) partially sensitive to NBT ($15.9 \pm 1.4\%$ block by 1 μM NBT) and has a proline at position 198, as does $\alpha 2$. Changing pro198 of $\alpha 4$, to gln as in $\alpha 3$, increases NBT sensitivity ($87.4 \pm 6.8\%$ block by 1 μM NBT). These results show thr143 to be a major determinant of the NBT sensitivity of $\alpha 3$ and provide additional evidence of a role for gln/pro198 in determining NBT sensitivity.

Both NBT and dihydro- β -erythroidine (DH β E) are useful probes of the β subunit contribution to competitive antagonist sensitivity. At ACh concentrations approximating the EC₂₀, $\alpha 3\beta 2$ is 50 fold more sensitive to DH β E (IC_{50} = 400 nM) than $\alpha 3\beta 4$ (IC_{50} = 20 μM). 3 μM DH β E effectively blocks $\alpha 3\beta 2$ ($90 \pm 4.6\%$ block) but has little effect on $\alpha 3\beta 4$ ($13.1 \pm 5.1\%$ block). Substituting in the first 105 N-terminal amino acid residues of $\beta 4$ into $\beta 2$, results in a subunit that forms receptors with $\beta 4$ -like ligand sensitivity, i.e., insensitive to both 100 nM NBT and 3 μM DH β E. Substituting in the first 58 residues of $\beta 4$ resulted in intermediate sensitivity to NBT and DH β E ($80 \pm 1.7\%$ block by 100 nM NBT; $71 \pm 6.6\%$ block by 3 μM DH β E). Thus, amino acid residues within two distinct sections of the β subunit (1-58 and 58-105) are involved in determining sensitivity to competitive antagonism.

CHARACTERIZATION OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS AS TARGETS FOR NOVEL CNS DRUGS. Lloyd G.K., McDonald, I., Harpold, M., Ellis, S.B., K., Elliott, K., Johnson, E.C., Chavez-Noriega, L., Velicelebi, G., Stauderman, K., Rao, T.S., Sacaan, A., Reid, R., and Menzaghi, F. SIBIA, 505 Coast Blvd. South, Suite 300, La Jolla, CA 92037.

There is increasing evidence that combinations of nicotinic acetylcholine receptor (nAChR) α and β subunits encoded by at least nine different genes result in different CNS nAChRs, which very likely regulate different physiological and functional activities (see accompanying abstracts). Thus, compounds specific for individual nAChR subtypes are likely to have highly selective therapeutic endpoints. We have investigated subtype and functional selectivities using an integrated approach involving various biochemical and functional assays, including intracellular calcium levels in recombinant mammalian cells expressing human nAChRs, nicotinic agonist ligand binding to rat brain membranes, neurotransmitter release from rat brain regions, electrophysiology of specific human nAChR expressed in *Xenopus* oocytes, rotation in unilaterally 6-OH dopamine-lesioned rats, and activity in the rat tail flick assay.

Using this portfolio we have observed that changes to the structure of nicotine can alter *in vitro* and *in vivo* activity which is indicative of nAChR subtype selectivity. This is exemplified by studies comparing nicotine and 5-bromonicotine.

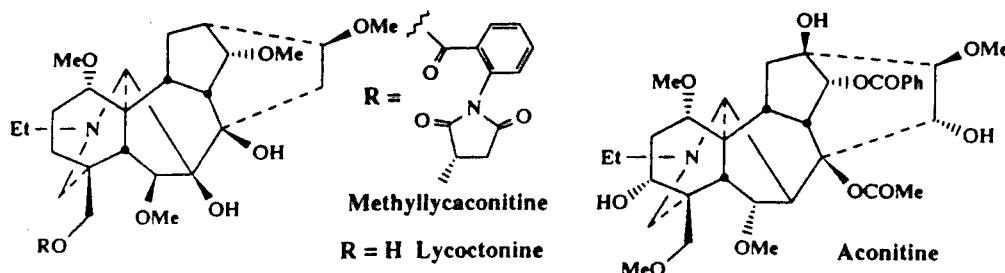
Compound	Intracellular Calcium Elevation $\alpha 3\beta 4$ in HEK 293 cells	Neurotransmitter Release		<i>in vivo</i> Function		
		DA Striatum	NE Hippocampus	6-OHDA Turning	Tail Flick Analgesia	Cardio-vascular
Nicotine	Agonist	Agonist	Agonist	Ipsi	++	Hypertens. Tachycardia
5-BrNic	Antagonist	Agonist	Partial Agonist	Ipsi	+	Hypotens.

STRUCTURAL DETERMINANTS OF THE POTENCY AND SELECTIVITY OF METHYLLYCAONITINE FOR THE $\alpha 7$ nAChR

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Methyllycaconitine (MLA) is a hexacyclic norditerpenoid alkaloid isolated from various *Delphinium* and *Consolida* spp., but not found in *Aconitum*, despite its trivial name. MLA is a competitive antagonist of the nicotinic acetylcholine receptor (nAChR) with selectivity for neuronal α -bungarotoxin (α -BgTX)-sensitive nAChR. Furthermore, MLA is only a weak ligand at muscle nAChR, making MLA more discriminating than α -BgTX (Wonnacott *et al.*, *Methods in Neurosciences*, 1993, 12, 263-275). Structurally, MLA is a norditerpenoid ester of a substituted 2-succinimidobenzoic acid. Hydrolysis of the ester bond affords the parent neopentyl-like alcohol lycoctonine. This alcohol has significantly reduced potency, 10^{-5} M compared to 10^{-9} M for MLA, at brain α -BgTX binding sites. However, the selectivity for this nAChR subtype is retained with lycoctonine. These alkaloids are structurally related to the sodium channel activator aconitine. We have synthesized the corresponding hybrid MLA-aconitine ester (Hardick *et al.*, *Tetrahedron Letters*, 1994, 35, 3371-3374). This novel hybrid analogue displayed potency comparable to that of MLA for nAChR. Thus, motifs within the highly substituted norditerpenoid core confer $\alpha 7$ selectivity, whereas the anthranilate ester moiety is crucial for nanomolar affinity.

Acknowledgements: We thank the Wellcome Trust (036214), BVLP is a Lister Institute fellow.



P8.11

MAPPING AMINO ACID(S) RESPONSIBLE FOR THE PHARMACOLOGICAL DIFFERENCES OBSERVED BETWEEN $\alpha 7$ AND $\alpha 8$ HOMOMERS.

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Homomers of $\alpha 7$ and $\alpha 8$ subunits of chick α Bgt-sensitive neuronal AChRs expressed from cRNAs in *Xenopus* oocytes exhibit similar channel properties but contrasting pharmacological properties (Gerzanich et al., Mol. Pharmacol. 45: 212-220, 1994). $\alpha 8$ homomers are more sensitive to agonists than $\alpha 7$ homomers (e.g. EC₅₀ (μ M) : ACh 2 vs 112; nicotine 1 vs 7; cytosine 1 vs 18; tetramethylammonium 10 vs 800).

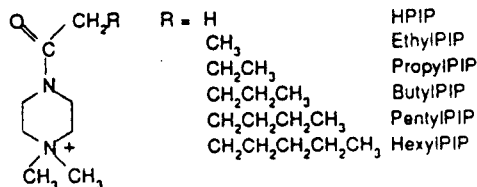
To delineate the amino acid residues responsible for these pharmacological differences, we have constructed $\alpha 7$ and $\alpha 8$ subunit chimeras as well as point mutants. A chimera consisting of the first 208 amino acid residues of the $\alpha 7$ subunit and the rest of the amino acid residues of the $\alpha 8$ subunit ($\alpha 7(208)/\alpha 8$), when expressed in oocytes, formed homomers whose pharmacological properties were indistinguishable from those of $\alpha 7$ homomers. A second chimera ($\alpha 7(115)/\alpha 8$) formed homomers whose properties were indistinguishable from those of $\alpha 8$ homomers. A third chimera ($\alpha 7(179)/\alpha 8$) formed homomers whose affinity for ACh was slightly higher (EC₅₀ \sim 30 μ M) than that for $\alpha 7$ homomers but distinctly different from that of $\alpha 8$ homomers.

Mutating the $\alpha 7$ subunit residue F187 to the $\alpha 8$ subunit residue Y187 did not alter the pharmacology of the $\alpha 7$ mutant homomers. Thus it appears that the five amino acid residues that are different between these two subunits within $\alpha 179$ and $\alpha 208$, contribute significantly to the pharmacological differences between $\alpha 7$ and $\alpha 8$ homomers. Further mutagenesis is in progress to verify this prediction.

P8.12

DIFFERENCES IN CHANNEL BLOCKADE FOR SKELETAL MUSCLE NICOTINIC ACETYLCHOLINE RECEPTOR VERSUS THE NEURONAL HOMOMERIC $\alpha 7$ NICOTINIC ACETYLCHOLINE RECEPTOR. Vazquez, R.W. and Oswald, R.E. Department of Pharmacology, Cornell University, Ithaca, NY 14853 USA.

We have previously shown using single channel recording that a series of cholinergic agonists with systematically varying structures can activate the mouse skeletal muscle nicotinic acetylcholine receptor (nAChR) with similar affinities, but at hyperpolarizing potentials, the channel blocking properties of these compounds differ dramatically and systematically (A.A. Carter & R.E. Oswald, *Biophysical Journal*, 65:840-851, 1993). The parent compound is 1,1-dimethyl-4-acetylpiperazine (HPIP), and the compounds that have been studied are shown in the figure below. The free energy for binding to the site for channel blockade decreases by 2.4 kJ/mole for the addition of each methylene on the acetyl carbon. We have begun a program combining specific site mutations of the mouse skeletal muscle nAChR and the neuronal homomeric $\alpha 7$ nAChR with the effects of channel blockade by the family of compounds shown below. Our initial two electrode voltage clamp experiments with wild type receptors expressed in *Xenopus* oocytes have revealed interesting differences between the muscle and neuronal receptors. As observed in BC₃H-1 cells, all six compounds can activate the mouse skeletal muscle nAChR but exhibit voltage-dependent channel blockade with the potency of the blockade increasing with increasing number of methylene groups. Results obtained with the homomeric $\alpha 7$ nAChR are qualitatively different. HPIP, EthylPIP and PropylPIP all exhibit minimal channel blockade except at hyperpolarizing potentials of greater than 100 mV. ButylPIP, PentylPIP, and HexylPIP all exhibit minimal apparent agonist activity. This seems to arise from a channel blockade rather than action at the ACh binding site because currents can be observed more readily at low agonist concentrations and low voltages. Thus, whereas the channel blockade by this series of compounds in skeletal muscle nAChR exhibits a continuous change in channel blocking properties with change in structure, the neuronal nAChR exhibits a strikingly discontinuous trend. Channel mutations of the skeletal muscle and neuronal nAChRs will be used to explore the differences in the properties of channel blockade.



P8.13

THE TRANSMEMBRANE DOMAINS OF THE NICOTINIC ACETYLCHOLINE RECEPTOR CONTAIN α -HELICAL AND β STRUCTURES. Strecker, A., Göme-Tschelnokow, U., Kaduk, C., Kaufmann, R.[#] and Hucho, F. Institut für Biochemie, Freie Universität Berlin, Berlin, Germany and [#]Institut für Lasermedizin, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

The transmembrane domain of the nicotinic acetylcholine receptor (nAChR) is responsible for opening the integral membrane channel following binding of an agonist molecule to the extracellular binding site. However, conflicting results exist concerning the secondary structure of the (nAChR) within the membrane spanning domain. While folding prediction algorithms propose exclusively α -helical folding, various approaches have resulted in β -sheets being located in the membrane. In this work, receptor-rich membranes were treated with proteinase K to cleave off water-accessible protein parts. The resulting membranes contained 25-30% of the applied protein and less than 1% of the original α -Bungarotoxin binding sites. Cleavage of intravesicular protein domains was demonstrated by disappearance of the stoichiometrically associated intracellular 43kD (ν_1) protein. The sample was shown by FTIR spectroscopy to consist of 50% α -helical-, 40% β -sheet- and 10% random structure. α -helical structures in these membranes were shown by linear dichroism to be oriented with respect to the membrane plane. Preliminary results with MALDI-MS and post-source-decay analysis indicate that α -M3 is continuously embedded in the membrane and longer than predicted from folding algorithms

P8.14

INBRED MOUSE STRAINS WITH DIFFERING NUMBERS OF HIPPOCAMPAL α -BUNGAROTOXIN BINDING SITES SHOW CORRELATED ABILITY TO GATE EVOKED RESPONSES TO PAIRED AUDITORY STIMULI. Stevens, K.E., Freedman, R., Marks, M.J., Collins, A.C., Leonard, S., Hall, M. and Rose, G.M. Department of Psychiatry, University of Colorado Health Science Center, Denver, CO 80262, USA

The midlatency auditory evoked potential recorded to the second of a closely-spaced (0.5 sec) pair of tones is reduced, as compared to the first, in unmedicated rats and normal humans. Schizophrenics and psychotomimetic-drug-treated rats routinely produce responses of similar magnitude to both stimuli; thus, they do not gate. Recently, a schizophrenia-like loss of hippocampal gating has been shown in rats following central administration of d-tubocurarine or α -bungarotoxin (α -BTX). Several strains of inbred mice have varying numbers of hippocampal α -BTX-binding sites. Mice from 9 strains were studied to determine whether the hippocampal α -BTX-sensitive nicotinic receptor is involved in modulation of auditory gating. Mice from the ST/b, C3H, C57/BL, AKR, BUB/J, BUB/IBG, BALB, DBA/2IBG and DBA/1J strains were assessed for ability to gate to paired auditory stimuli in an anesthetized paradigm. When the level of whole hippocampus α -BTX binding was correlated with TC ratio (the measure of gating) a significant correlation was obtained ($r=-0.72$, $p<0.05$). Thus, mice with more α -BTX sites gate better. Correlation of striatal α -BTX binding with gating was not significant. Autoradiography for hippocampal α -BTX binding and *in situ* hybridization for mRNA for the nicotinic receptor subunit $\alpha 7$ (thought to be the α -BTX binding subunit) were performed on 4 of the strains of mice. α -BTX binding in the CA3 region showed significant correlation with TC ratio ($r=-0.89$, $p<0.05$) as did level of mRNA for the $\alpha 7$ subunit ($r=-0.93$, $p<0.05$). Neither the CA1 or dentate gyrus showed a significant correlation of TC ratio with either measure. As a further test of nicotinic modulation of gating, mice from the DBA substrains were injected with 3 mg/kg, nicotine, sc. The nicotine produced transient periods of normal gating in both substrains of mouse. These data suggest that nicotinic α -BTX sensitive receptors, specifically those in the CA3 region of the hippocampus, may play a role in the modulation of hippocampal auditory gating.

P8.15

STRUCTURE-ACTIVITY RELATIONSHIP FOR THE ACTIONS OF ACRIDINE ARAPHANES ON CHOLINERGIC RECEPTORS. Shaw, K.-P. Dept. Bio. and Anatomy: Dept. Pathology, National Def. Med. Center. Tapei, Taiwan, China.

Acridine araphanes (Himelet al., *Science* 205:1277, 1979; Taylor et al., *Mol. Pharmacol.* 45:74, 1979) are structurally related to 9-amino-1,2,3,4-tetrahydroaminoacridine (THA), a drug that has been approved by the FDA for the treatment of Alzheimer's disease. The objective of the present study was to determine the structure-activity relationship of acridine araphanes at nicotinic as well as muscarinic receptors by using biochemical assays and molecular modeling. 1,2-PAA (1,2-isopropyl acridine araphane), 1,3-PAA (1,3-propyl acridine araphane), 1,4-BAA (1,4-butyl acridine araphane), 1,5-PeAA (1,5-pentyl acridine araphane), 1,6-HeAA (1,6-hexyl acridine araphane), 1,7-HepAA (1,7-heptyl acridine araphane), and 1,9 NAA (1,9-nonyl acridine araphane) inhibited the binding of [³H]-N-methylscopolamine to muscarinic receptors in the brain stem with IC₅₀s of 16, 5, 1.1, 8.9, 14, 56, and 1.4 nM, respectively, and inhibited the binding of [¹²⁵I]α-BGT to *Torpedo* nicotinic receptors with IC₅₀s of 0.8, 0.1, 0.68, 0.99, 0.11, 0.36, and 0.1 μM, respectively. The acridine araphanes showed higher affinity than THA and mono-amino acridines for the cholinergic receptors. The structure-activity relationship was then established by molecular modeling. In the series of acridine araphanes, increasing the number of carbon atoms (n) from two to four increased the affinity of the resultant compound to the muscarinic receptors. Further elongation of the alkyl chain up to 7 carbons decreased the affinity of the drugs. However, when the number of carbons was increased to 9, the affinity of the resultant compound was similar to that of 1,4-BAA. The full geometry optimization using the semi-empirical program AM1 showed that when n=2-3, the aromatic rings were relatively parallel, and that when n=4 or 9, the aromatic rings are almost 180° apart. The predictable conformation of the acridine araphanes and the distinct interactions of these compounds with nicotinic and muscarinic receptors suggest that these drugs might be useful for a better understanding of the stereochemical structure of these receptors. *Support:* U.S. Army Med. Res. & Develop. Comm. Contract DAMD 17-88C-8119.

P8.16

PH DEPENDENCE OF METHYLCARBAMYLCHOLINE BINDING TO RAT BRAIN AND TORPEDO MUSCLE NICOTINIC RECEPTORS. Mahnir, V.M., and Kem, W.R. Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, FL 32610, USA.

Although the influence of pH upon nicotinic ACh receptor ion channel properties is well known, few studies of the pH dependence of binding to the ACh recognition site have been reported. Before investigating the binding of weakly basic agonists, we decided to assess the influence of pH upon the binding of [³H]-Methyl-carbamylcholine (MCC), which is permanently ionized under physiological conditions. Thoroughly washed rat whole brain and *Torpedo californica* electric organ membranes were exposed to tritiated MCC (10 nM) for 2 hr at room temperature in a balanced saline buffered with MES-HEPES; unbound ligand was removed by quickly washing membranes on GF/C filters with ice-cold saline. At 10 nM, MCC binding to rat brain is largely due to the relatively abundant Alpha4-Beta2 subtype which displays high affinity for this radioligand. Non-specific binding was determined in the presence of 0.1 mM carbamylcholine. Although non-specific MCC binding in both membrane preparations was not influenced by pH over the 6.0-7.8 range investigated, specific binding to both types of nicotinic receptor was significantly enhanced by increasing pH. For the rat brain receptor, binding was progressively enhanced by 129% (5 experiments) as the pH was increased from 6.0 to 7.8, the effect approaching a maximum at the highest pH. In contrast, the *Torpedo* receptor binding of MCC decreased slightly as pH was increased from 6.0 to 7.0, then displayed a more pronounced increase (relative to the rat brain high affinity receptor) in binding as pH was raised from 7.0 to 7.8; this effect was not complete at pH 7.8. Binding was enhanced by 290% (3 experiments) relative to that measured at pH 7.0. Several tyrosyl groups are known to contribute to the binding of quaternary ammonium compounds in muscle (Sine et al., 1994) and neuronal receptor subtypes. Ionization of one or more of these sidechains might conceivably cause the enhancement in MCC binding affinity we observed. Identification of receptor amino acid sidechains involved in pH-dependent binding of MCC should be possible using mutagenesis, affinity labelling, and other techniques. (Partially supported by Taiho Pharmaceutical Co., Ltd.)

P8.17

SMALL CELL LUNG CARCINOMA (SCLC) DERIVED FROM A MYASTHENIA GRAVIS PATIENT EXPRESSES NICOTINIC ACETYLCHOLINE RECEPTORS OF BOTH MUSCLE- AND NEURONAL-TYPES. Sciamanna, M. A., Griesmann, G. E., Wieben, E.,* and Lennon, V. A. Depts. of Immunology, Neurology, Laboratory Medicine and Pathology, and Biochemistry*, Mayo Clinic, Rochester, MN, U.S.A.

Factors that initiate and maintain the autoimmune response in myasthenia gravis (MG) are not yet known. The high frequency of MG in patients who have thymoma suggests that autoimmunity in some cases may represent anti-tumor immune responses initiated by aberrant autoantigen expression. Other tumors, including SCLC, have been reported in association with MG. We recently established a SCLC line (SCC-37) from a patient with clinical and serological evidence of MG (Soc Neurosci Abstr 19:199, 1993). Unlike other SCLC lines, carbachol induces an influx of $^{22}\text{Na}^+$ in SCC-37. This response is inhibited by curare and α -bungarotoxin (α -BTx). Receptors for α -BTx in SCC-37 are comparable in concentration to adult muscle tissue (250 fmol/g tumor). Detergent-solubilized receptors sediment as pentamers in a density gradient, and are immunoprecipitable by anti-muscle-type nAChR IgG, a property that distinguishes them from neuronal-type α -BTx-receptors. RT-PCR analysis of total SCC-37 RNA generated a full-length mature α_1 subunit cDNA identical in sequence to human muscle nAChR. Northern analysis detected nAChR α_1 in SCC-37 and in the TE-671 human skeletal muscle line, but not in SCC-9, a prototypic SCLC line. These data confirm the expression of muscle-type nAChRs in SCC-37.

Certain SCLC lines have been reported to express neuronal-type nAChRs (FEBS 312:66, 1992). The neuronal nicotinic agonist, cytisine, induces an influx of $^{45}\text{Ca}^{2+}$ in SCC-37, but not in TE-671. Inhibition of this response by both α -BTx and the neuronal nAChR antagonist, methyllycaconitine (MLA), implies activation of an α_7 -type neuronal nAChR. By limiting dilution, we selected a subclone of SCC-37 (A9) in which carbachol induces negligible $^{22}\text{Na}^+$ influx but cytisine induces a $^{45}\text{Ca}^{2+}$ influx response that is inhibited by both α -BTx and MLA. Binding of ^{125}I - α -BTx to the parental SCC-37 line and its A9 subclone (0.53 and 0.35 fmols/ 10^5 cells, respectively) is inhibited -40% by 1 nM MLA. These results are consistent with co-expression of functional nAChRs of both muscle-type (α_1) and neuronal-type (α_7) in SCC-37, and with a predominance of neuronal-type (α_7) expression in its A9 subclone. Supported by C. T. Fernie Fund and Sosin Postdoctoral Fellowship from the National MG Foundation.

P8.18

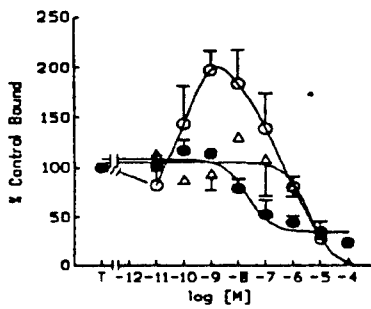
ACTIONS OF NICOTINIC AGONISTS ON THE INTERMEDIOLATERAL NUCLEUS NEURONS IN THE NEONATE RAT SPINAL CORD *IN VITRO* Bordey, A., Trouslard, J. and Feltz, P. Laboratoire de Physiologie Générale (URA CNRS 1446); 21, rue R. Descartes; 67084 Strasbourg Cedex; France

We studied the responses of specific nicotinic agonists, DMPP (Dimethyl-4-phenyl piperazinium iodide) and nicotine in thin (250 μM) transverse slices of the thoracic and lumbar spinal cord of neonatal rats (P0-P8) using the whole-cell recording configuration of the patch-clamp technique. We recorded two types of neurons in the Intermediolateral cell column (IML): one possess an A current and is assumed to be sympathetic preganglionic neurons (SPNs) on the basis of their morphology, the other type has no A current and is proposed to be interneurons (Inokuchi et al., J. Auton. Nerv. Syst. 43, 1993). In SPNs, DMPP had two effects: 1) a short term effect consisting of the induction of a postsynaptic current and 2) a long term effect which is the induction of fast excitatory and/or inhibitory currents (respectively EPSCs and IPSCs) lasting 20-30min after the end of DMPP application. At -50mV, a pressure application of DMPP induced an inward current accompanied by an increase in conductance and characterized by two phases: a first transient peak (-181.3 \pm 100pA, n=3) showing a fast desensitization followed by a plateau phase (-77.8 \pm 14 pA, n=7). It reversed at 0mV and was not abolished by bath perfusion of lanthanum (200 μM). Concerning the long lasting effect, nicotinic agonists (1 mM, 10-20 sec) triggered the appearance of EPSCs and/or IPSCs at -50mV, both in SPNs and in interneurons. In SPNs, previous studies showed that these synaptic currents are respectively carried by glutamate and glycine. These results suggest that nicotinic receptors are located postsynaptically on SPNs and presynaptically on both glycinergic interneurons that project to SPNs and on glutamate-containing terminals that project either to SPNs or to unidentified neurons of the IML.

P8.19

INFLUENCE OF MELATONIN ON 1-[³H]-NICOTINE BINDING SITES IN THE RAT VAS DEFERENS. Carneiro R.C.G., Zago W.M., Markus R.P. Dep. Pharmacology, Institute of Biomedical Science - University of São Paulo, São Paulo, Brazil.

The contraction induced by acetylcholine in the prostatic portion of the rat vas deferens is due to the release of norepinephrine and ATP by stimulation of presynaptic nicotinic receptors located on adrenergic nerve terminals (Carneiro RCG and Markus RP, JEPT 255: 95, 1990). This contraction can be potentiated by endogenous or *in vitro* melatonin (MEL). The effect of MEL is not related to changes in postsynaptic reactivity and is dependent on the presence of hypogastric ganglion (Carneiro RCG et al, JEPT 259:614, 1991; Eur. J. Pharmacol *in press*). The aim of this study is determine if MEL modifies the 1-[³H] nicotine binding sites located in the adrenergic nerve terminals in the prostatic portion of the rat vas deferens. Vas deferens prostatic portions from rats (4 months, light-dark cycle 12:12h, sacrificed at 15:00h) were incubated in the absence or presence of MEL (100 pg/ml) for 4 hours. 1-[³H] nicotine binding sites were analysed by competition studies using nicotine as non-radioactive ligand. After the



incubation period membranes were prepared by two successive centrifugations (600 xg, 10 min, and 40,000 xg, 20 min 4°C) of a homogenate prepared in Hepes buffer with the following composition (mM): NaCl 120.0; KCl, 3.0; Na₂HPO₄ 8.0; KH₂PO₄ 1.5; Hepes 20.0; EDTA 2.0; PMSF 0.1; Iodoacetamide 1.0 and sucrose 320. The membranes were incubated with 1-[³H] nicotine (5-6 or 50-60 nM) for 2 hours at 4°C in the absence and the presence of nicotine (10 pM - 0.1 mM). The figure shows a low-dose hook effect for displacement of 5-6 nM of 1-[³H] nicotine from organs not incubated with MEL (O). This low-dose hook effect is missed using either higher doses of the labeled specie (50-60 nM; Δ) or membranes from tissues incubated with MEL (●), T (Total binding). The

lost of the low-dose hook effect comparing low and high concentrations of 1-[³H] nicotine suggests the existence of a positive cooperative binding in membranes from tissues obtained from rats sacrificed at 15:00 h. The positive cooperativity is probably correlated to non-functional receptors, since, at this hour of the day the tissue shows a very low responsiveness to acetylcholine. Even more, *in vitro* MEL, not only recovers the functional response, but also impairs the low-dose hook effect to 5-6 nM 1-[³H] nicotine.

Financial support: FAPESP 93/1846-5 Fellowships :RCGC, FAPESP, RPM, WMZ: CNPq.

P8.20

INFLUENCE OF THE HOUR OF THE DAY ON THE BINDING TO NICOTINE RECEPTORS AND FUNCTIONAL RESPONSE TO NICOTINE AND DMPP IN THE PROSTATIC PORTION OF THE RAT VAS DEFERENS. Markus R.P., Carneiro R.C.G., and Zago W.M. Department of Pharmacology, Institute of Biomedical Science, University of São Paulo, Brazil.

The prostatic portion of rat vas deferens shows an adrenergic-cholinergic axo-axonal interaction. Stimulation of presynaptic nicotinic receptors located in the adrenergic nerve terminals induces the release of norepinephrine and ATP that act as neurotransmitters. The maximal contraction induced by acetylcholine shows an hour of the day variation (Carneiro RCG et al, JEPT 259:614, 1991) due to direct melatonin action on sympathetic neurons (Carneiro et al, Eur. J. Pharmacol., *in press*). The aim of this study was to evaluate the hour of the day effect on the desensitization of the contractile response and 1-[³H]nicotine receptor binding displacement by DMPP and nicotine. Prostatic portions were removed from rats (4 months) maintained in light-dark cycle (12:12 h) sacrificed at 15:00 and 21:00 h. Isometric non-cumulative contractions for DMPP and nicotine (60 min interval among administration) were recorded as described before (Carneiro RCG and Markus RP, JEPT 255:95,1990). 1-[³H] nicotine binding sites were analyzed by competition studies using nicotine and DMPP as non-radioactive ligand. Membranes were prepared by two successive centrifugations (600 xg, 10 min, and 40,000 xg, 20 min 4°C) of homogenates prepared in Hepes buffer. The membranes were incubated with 1-[³H] nicotine (5-6 nM) for 2 hours at 4°C in the absence or the presence of DMPP (10 pM - 10 μM) or nicotine (10 pM - 100 μM). The maximal DMPP and nicotine contraction were not dependent on the hour that the animal was sacrificed. The pD₂ values for DMPP were not modified by the hour of the day (15:00, 4.20 ± 0.21, 6). However, for nicotine, a difference in pD₂ values (15:00, 3.80 ± 0.07 (13); 21:00 h, 4.20 ± 0.01 (16); p<0.01) was observed. Displacement studies showed an hour of day effect on nicotine, but not on DMPP effect. For nicotine a low-dose hook effect was observed when animals were sacrificed at 15:00 h, but not at 21:00h, suggesting a positive correlation between binding sites. Thus, the data suggest a correlation between the binding displacement and the functional response to nicotinic agonists depending on the light environmental cycle.

Financial support: FAPESP 93/1846-5; CNPq 500934/90-8 Fellowships: RCGC: FAPESP, RPM and WMZ: CNPq.

P8.21

EFFECT OF NICOTINE ON AMNESIA INDUCED BY ANTIMUSCARINIC AGENTS IN MOUSE PASSIVE-AVOIDANCE TEST

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Much evidence suggest the ability of nicotine, via a positive feed-back mechanism, to increase ACh release both in the CNS and in the periphery through stimulation of presynaptically localized nicotinic receptors (Beani et al., 1985; Bowman et al., 1988; Rowen and Winkler, 1984). Iwamoto (1989) described the possibility to prevent ACh releasing action by pretreatment with (-)-vesamicol, an agent that interferes with storage/or release of ACh. ACh is considered a primary neurotransmitter in mnemonic function; therefore the purpose of this research was to evaluate the role of nicotine in learning and memory processing. Nicotine was tested in the mouse in a modified one trial pass-through passive avoidance test consisting of a painless punishment (fall into cold water, 10°C). Mouse cognitive processes had been impaired by dicyclomine (2 mg/kg i.p.), pirenzepine (0.1 µg/mouse i.c.v.) or scopolamine (1 mg/kg i.p.) administered immediately after the training session. In this test nicotine at the dose 0.1-0.5 mg/kg s.c. and 0.3-1mg/mouse i.c.v., injected 10 min before the training session was able to protect animals from amnesia induced by antimuscarinic treatments. In the same range of doses nicotine was unable to improve learning in mice devoid of memory impairment. Effective doses of nicotine failed to affect rota-rod performance and spontaneous locomotor activity.

This study supports the possibility of preventing cognitive deficits through activation of nicotinic autoreceptors by using nicotine at lower doses than those responsible for a post-synaptic effect. It could represent a novel therapeutic strategy for the treatment of dementia.

This work was supported by grants from MURST and CNR

P8.22

NEW INSIGHTS INTO THE CHARACTERIZATION OF THE SECOND PATHWAY FOR NICOTINIC RECEPTOR ACTIVATION. Aracava, Y., Teixeira, C.M.S., Tano, T., and Nascimento, B.S. Lab. Mol. Pharmacol., UFRJ, Rio de Janeiro, RJ 21944, Brazil.

It has been demonstrated that (-)physostigmine, certain polyamines, and 4-methylpyrazole (4-MP) activate nicotinic receptors (nAChRs) of *Torpedo* electroplax, frog muscles, and hippocampal neurons by binding to a nAChR site distinct from that for ACh (Pereira et al., *J. Rec. Res.*, 13:100, 1993). Activation of nAChRs via this novel binding site is insensitive to blockade by competitive nicotinic antagonists and to desensitization induced by high concentrations of ACh, whereas it can be inhibited by the nAChR-specific monoclonal antibody FK1. The question was raised how open-channel blockers (e.g., atropine) and allosteric inhibitors of the nAChR activity (e.g., nortriptyline) could affect the nAChR activity when the receptor channel is activated via the newly described site. Single-channel currents elicited by 4-MP (10 µM) were studied in interosseal fibers dissociated from the hind foot of *L. oxellatus* under cell-attached configuration. Nortriptyline (1 µM) abolished the agonist effect of either ACh or (+)-anatoxin-a, but did not alter that of 4-MP. In contrast, atropine (2-10 µM) had similar effects on currents activated by either ACh or 4-MP. In the presence of atropine, the lifetime of 4-MP- or ACh-activated channels was significantly shortened; short-lived, isolated single-channel currents could be recorded, indicating that atropine induced a rather stable open-channel blockade. Thus, desensitization induced by either high concentrations of ACh or allosteric antagonists such as nortriptyline seems to specifically uncouple ACh binding from channel gating. On the other hand, open-channel blockade occurs independently of the pathway through which the nAChR channel is gated. *Support:* Mol. Pharmacol. Train. Prog. UFRJ/UMAB.

SPECTRIN AT SYNAPSES FORMED BY HIPPOCAMPAL NEURONS. L.A. Martin,
B. E. Alger, and R.J. Bloch. Department of Physiology, University of Maryland School of
Medicine, Baltimore, MD 21201

The cytoskeletal protein, spectrin, is involved in the genesis and organization of postsynaptic clusters of nicotinic acetylcholine receptors in muscle. We have been studying hippocampal cells in tissue culture to learn if members of the spectrin family are involved in synaptogenesis in the central nervous system. We prepared subunit-specific polyclonal antibodies to β -spectrin (β I), β -fodrin (β II), and α -fodrin (α II). Extracts of rat hippocampi were analyzed by immunoblotting to determine when during postnatal development the three subunits were expressed. Both fodrin subunits (α II and β II) were expressed at all ages, whereas β I expression increased substantially early in postnatal life, when synaptogenesis in the hippocampus occurs. Immunoblots of cerebral neurons and astrocytes showed β I expression primarily in neurons, and α II and β II in both neurons and astrocytes. Immunofluorescence studies of spectrin in cultured hippocampal cells confirmed the presence of β I primarily in neurons and α II and β II in both neurons and astrocytes. Within neurons, β I was found in neuronal cell bodies and dendrites, whereas β II and α II were localized in axons, cell bodies and dendrites. Preliminary studies suggested that, in neurons with spines, β I, but not α II or β II, was concentrated in spine heads, whereas α II, but neither β subunit, was present in spine necks. The subcellular localization and developmental regulation of β I-spectrin is consistent with the hypothesis that β I participates in postsynaptic differentiation in the CNS.

Supported by grants from the NIH (NS 17282, NS 22010, NS 30219 and HD16596) and by a by training grant GM 08181.

*Third International Symposium
The Cholinergic Synapse:
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SUBTYPE -SELECTIVE CHOLINERGIC ION CHANNEL AGONISTS AS POTENTIAL ANTIPARKINSON AGENTS. Lloyd, G.K., McDonald, I.A., Vernier, J.-M., Elliott, K., Ellis, S.B., Sacaan, A., Rao, T.S., Chavez-Noriega, L., Johnson, E.C., Velicelebi, Mengaghi, F., and Harpold, M. SIBIA, 505 Coast Blvd. South, Suite 300, La Jola, CA 92037.

The SIBIA Cholinergic Ion Channel (CIC) program is dedicated to the discovery and development of CIC receptor subtype-specific agonists for the treatment of CNS disorders. The results of *in vitro* and *in vivo* assays of a series of compounds being developed in SIBIA's CIC/Parkinson's Disease Program, reveal that S-1663 has a high specificity for the agonist recognition site in the CIC as compared to other members of the ligand-gated ion channel superfamily. S-1663 induces release of dopamine (DA) from striatal slices with a lesser effect on norepinephrine (NE) release from hippocampal slices. Furthermore, S-1663 is subtype selective for human CIC recombinants with agonist activity at $\alpha 3\beta 4$ but not at other neuronal recombinants, muscle or ganglion subtypes. *In vivo*, in rats, S-1663 induces striatal DA release as determined by ipsilateral rotations in unilaterally 6-hydroxydopamine lesioned rats; this activity is reversed by mecamylamine, a specific CIC antagonist. S-1663 reverses haloperidol-induced catalepsy, an animal model for extrapyramidal motor dysfunction. These observations, together with the available literature on nicotine, Parkinson's and Alzheimer's diseases indicate that specific CIC subtype agonists are potentially useful for treating both the motor and cognitive aspects of Parkinson's disease.

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Warner-Lambert Company (Parke-Davis Pharmaceutical Research)



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AT BALTIMORE

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Cover: Superimposed micrographs showing labeling for presynaptic $\text{Na}^+/\text{Ca}^{2+}$ exchangers (green) and postsynaptic nAChRs (red) at a neuromuscular junction.

Inset: Enlargement of a micrograph of an FK1-labeled cultured hippocampal neuron.