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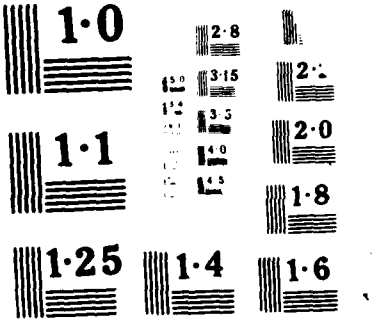
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IMPROVEMENTS IN THE METHODOLOGY FOR ANALYZING RECEPTOR SUBTYPES AND NEURONAL POPULATIONS AFFECTED BY ANTICHOLINESTERASE EXPOSURE

Annual Summary Report

James K. Wamsley

November 14, 1984

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Conditions have been defined which provide a means of selectively labeling subtypes of muscarinic receptors. The so-called M_1 receptor population can be labeled with tritiated pirenzepine, while the receptor population labeled with tritiated quinuclidinyl benzilate (QNB) but not labeled with pirenzepine represents the M_2 receptor population. High and low affinity states of the receptors have also been defined on the basis of agonist displacement of antagonist binding. The high affinity state of the M_2 receptor can be shifted to a low affinity state by including guanine nucleotides in the incubation medium. This guanine nucleotide shift has now been shown in microscopic regions of the brain where the M_2 receptor sites predominate. The high affinity state of the M_2 receptor can be labeled directly with tritiated cis-methyldioxolane (CD). There is some indication that tritiated CD may also be labeling some high affinity M_2 sites. Both the M_1 and M_2 receptor populations undergo axonal transport and the affinity states of these receptors

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are altered by neurochemical and neurosurgical lesions. Radioactive standards have been developed in this laboratory which provide a means of quantitating the femtomoles of receptor bound with each ligand in microscopic regions of the brain. These standards also make it possible to quantitate the amount of receptor undergoing axonal transport in both the brain and periphery. The technology has also been devised to directly localize nicotinic cholinergic receptors using tritiated nicotine. A method has been developed which allows the localization of cholinergic terminals using autoradiographic identification of choline uptake sites with tritiated hemicholinium-3. It is now possible to localize several peptide receptors associated with cholinergic function including receptors for thyrotropin-releasing hormone (TRH) and somatostatin. The receptor autoradiographic technique has also been carried beyond the receptor level of localization by using compounds to label adenylate cyclase and the GTP binding protein. This methodology should provide an elegant means of determining how anticholinesterase exposure has affected these many parameters of cholinergic nerve function.

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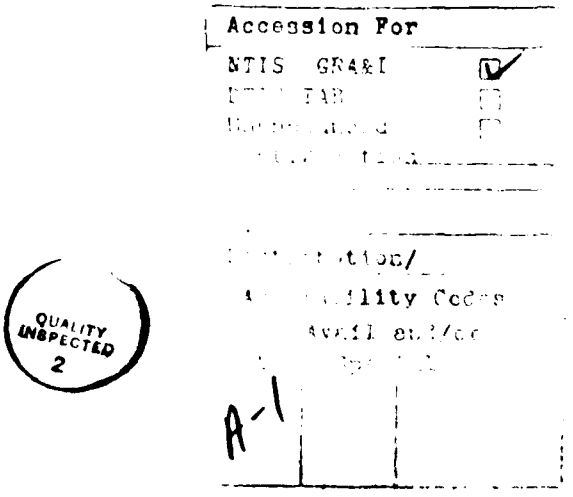

Summary

During the second year of this research project, several technological advances have been made which will provide a better means for determining how acute and chronic anticholinesterase exposures affect the regulation and transport of cholinergic receptors in the central nervous system. The conditions for obtaining selective labeling of the subtypes of muscarinic cholinergic receptors have been defined. The differential distribution of M_1 versus M_2 receptors has been described in several individual brain regions by comparing binding of tritiated pirenzepine ([^{3}H]-PZ) to that of tritiated quinuclidinyl benzilate ([^{3}H]-QNB). High and low affinity states for agonist binding have also been shown for these two receptor subtypes, and the high and low affinity states of the M_2 receptor have been mapped in many brain regions. The guanine nucleotide sensitivity of the M_2 receptors has been shown, and the ability to shift the high affinity state to the low affinity conformation has been demonstrated in several discrete microscopic regions of the brain. These high affinity conformations of the M_2 receptor can be labeled directly with tritiated cis-methyldioxolane ([^{3}H]-CD) using conditions defined in this report. Some of the high affinity sites are labeled with [^{3}H]-CD (i.e., those in the dorsal horn of the spinal cord) which suggests that CD is also capable of recognizing some high affinity states of the M_1 receptor.

A major advance in this laboratory in the past year is the development of radioactive standards which provide a means of quantitating the femtomoles of ligand bound per milligram of tissue in individual regions of the brain. It is thus now possible to determine the density of M_1 and M_2 receptors which are being transported in various pathways of the brain. It is also possible to determine how and to what extent these receptor subtypes are affected by acute and chronic exposure to anticholinesterase agents. The effects of these drug treatments on the parameters outlined above will be determined in subsequent parts of this study. We have also defined the conditions for obtaining selective labeling of the nicotinic cholinergic receptor using tritiated nicotine. This may be important, since the binding attributable to the presence of nicotinic cholinergic receptors differs to some extent from that originally reported for the nicotinic receptor on the basis of alpha-bungarotoxin binding. The effects of anticholinesterase exposure on the density and distribution of muscarinic receptor subtypes will thus be compared to its effects on the nicotinic cholinergic receptor population.

We had originally proposed to localize and quantitate cholinergic neurons on the basis of acetylcholinesterase staining using microdensitometric techniques and to assess the damage caused by anticholinesterase treatment on the identified cholinergic neurons. Another important advance made during the second year, related to this goal, was the definition of parameters for using tritiated hemicholinium-3 to provide the first autoradiographic localization of cholinergic terminals. This radioactive ligand binds to choline uptake sites present on the cholinergic terminal; therefore, its density and distribution, as defined by quantitative computer-assisted microdensitometry, reflect the density of cholinergic innervation in that area. It is possible with this methodology to quantitate the density of choline uptake sites, which may be altered to some extent by the drug environment or neuronal activity.

Various drug exposures may also affect the cholinergic terminal beyond the receptor level. Another asset to the present study would be the ability

to examine the coupling of the receptor to the second messengers found in cholinoceptive cells. An important advance in this reporting year was the definition and localization of radioactive ligands which selectively label the enzyme adenylate cyclase with tritiated forskolin and the GTP binding protein with gamma-thio-guanosine triphosphate. Approval for receipt of chemical surety materiel has recently been obtained so the proposed experiments on anticholinesterase-treated animals can be initiated. We are thus in a position to determine how chronic exposure to anticholinesterase agents affects the many components of muscarinic cholinergic activity in the central nervous system.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH)78-23, Revised 1978).

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Introduction

It is now possible to use receptor autoradiographic techniques to discriminate muscarinic cholinergic receptors in the brain. Conditions have been established for obtaining specific labeling of each receptor subtype and, through the use of appropriate standards, the density of these sites can now be quantified in individual microscopic regions of the brain.

Two subtypes of muscarinic cholinergic receptors have been described on the basis of several criteria (1-3). The binding to these two receptor sites is compatible with the concept of two distinct muscarinic receptor subtypes, the M_1 and M_2 receptors (4-6). Conditions have been defined which allow selective labeling of M_1 and M_2 receptor types with tritiated quinuclidinyl benzilate ([^{3}H]-QNB) and tritiated pirenzepine ([^{3}H]-PZ). Those muscarinic receptors labeled with [^{3}H]-PZ represent the M_1 muscarinic cholinergic receptor subtype. Those receptor types labeled with [^{3}H]-QNB but not with [^{3}H]-PZ represent the M_2 receptor subtype. QNB is capable of recognizing both sites with equally high affinity, whereas PZ labels only the M_1 receptor subtype. Making use of these properties, we have labeled serial sections of rat brain with either [^{3}H]-QNB or [^{3}H]-PZ (7-10).

Agonist (carbachol) displacement of bound [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) was shown earlier to reveal a subpopulation of high affinity muscarinic agonist sites (11). These sites can now be localized and quantitated through the use of autoradiographic techniques. We have also successfully labeled the high affinity M_2 receptor directly with the muscarinic agonist cis-[^{3}H]-methyldioxolane ([^{3}H]-CD) (12).

Using the conditions established for preferentially labeling M_1 and M_2 receptor subtypes with [^{3}H]-QNB and [^{3}H]-PZ, we attempted to establish that both of these receptor subtypes undergo axonal transport in the central and peripheral nervous systems. The difficulty in testing this possibility resulted because both [^{3}H]-QNB and [^{3}H]-PZ labeled sites accumulate adjacent to a lesion placed on either the vagus or sciatic nerve. Two possible interpretations can be made of these results: either only the M_1 receptor subtype was transported, or both the M_1 and M_2 receptor subtypes were transported. To adequately determine whether both of these receptor subtypes were being transported necessitated the quantitation of the subtypes relative to the total amount of receptor accumulating adjacent to the lesions. We developed tritiated standards which provide a means of achieving such quantitation.

In collaboration with Dr. Edythe London in the Neuropharmacology Section of the Addiction Research Center at the National Institute on Drug Abuse, my laboratory has successfully defined the conditions for labeling the nicotinic cholinergic receptor of the brain with L-[^{3}H]-nicotine (13).

In collaboration with Dr. Henry Yamamura of the Department of Pharmacology at the University of Arizona, we have recently developed (14) appropriate binding conditions for specific labeling of cholinergic nerve terminals with [^{3}H]hemicholinium-3 ([^{3}H]HC-3). This radioactive ligand selectively binds to choline uptake sites on the cholinergic nerve terminals. Quantitation and localization of the density and distribution of these sites thus reflects the concentration of cholinergic nerve terminals in various regions of the brain and defines the density of choline uptake sites on those nerve terminals.

It is increasingly apparent that many peptides may serve as neuromodulators of neuronal activity at synapses involving the more classical neurotransmitters found in the brain. Indeed, one way that

anticholinesterase exposure could affect cholinergic nerve function is through alteration of the neuromodulators and/or their receptors in the cholinergic pathways. Therefore, to adequately examine the possibility that anticholinesterase exposure affects these neuromodulators, it will be necessary to examine the receptors for these substances in cholinergic pathways before and after exposure to the anticholinesterase agents.

We have recently worked out the binding conditions for specific labeling of somatostatin receptors (15) and receptors for thyrotropin-releasing hormone (TRH) (16). It has been shown in other studies that these two peptides may be involved in the regulation of cholinergic nerve function (17,18).

Another possible effect of exposure to anticholinesterase agents concerns the coupling of the receptors to their second messengers within the cells. Both guanylate cyclase and adenylate cyclase are thought to affect coupling in cholinergic systems. Previous research involved the radioimmunoassay of cyclic nucleotides generated in response to challenge with a muscarinic agonist. In addition, we are now able to localize and quantitate the density of adenylate cyclase (19) and of guanine nucleotide binding protein (Gehlert, Yamamura and Wamsley, in preparation) in specific neuronal populations in the brain, using the same type of technology.

Methods and Results

I. Subtypes of Muscarinic Receptors.

Initial biochemical experiments were performed in order to identify appropriate parameters which would provide high ratios of specific to nonspecific binding (high signal-to-noise ratios) (20). Sections to be labeled with [^{3}H]-QNB were incubated for 120 minutes at room temperature in Krebs phosphate buffer (pH 7.4) containing 1 nM [^{3}H]-QNB (specific activity 33 Ci/mmol, Dupont NEN; Boston, MA). To label with [^{3}H]-PZ, serial tissue sections were preincubated for 30 minutes in the same buffer containing 20 nM [^{3}H]-PZ (specific activity 75 Ci/mmol, Dupont NEN; Boston, MA). Both sets of slides were given two 5 minute rinses in fresh buffer at 4°C and air-dried. All of the labeled slide-mounted tissue sections were then apposed to sheets of LKB Ultrofilm (LKB Instruments; Rockville, MD) in X-ray cassettes, and the film was exposed for 2 weeks to 2 months at 4°C (21,22). The latent images developed on the tritium-sensitive film were then photographed and analyzed using a Leitz Orthoplan microscope (Leitz; West Germany) equipped with an Orthomat camera system and a DADS Model 560 computerized microdensitometry system.

The muscarinic receptors in the ventral horn of the spinal cord, cerebellum, nucleus tractus solitarius, facial nucleus, lamina IV of the cerebral cortex, and the nucleus of the diagonal tract were labeled with [^{3}H]-QNB but not with [^{3}H]-PZ. These results indicate the presence of M_2 receptors in the identified areas. These receptors are similar with respect to [^{3}H]-QNB and [^{3}H]-PZ binding to those described in the heart, parotid, and lacrimal glands and in the ileum. The muscarinic receptors in the dorsal horn of the spinal cord, striatum, hippocampus and most of the other laminae of the cerebral cortex were labeled with both [^{3}H]-QNB and [^{3}H]-PZ. The latter subset of receptors is thus similar to the ganglionic receptors and are termed the M_1 type.

To preferentially displace binding to the high affinity sites we competed 100 μM carbachol against the binding of [^{3}H]-NMS (55 Ci/mmol,

Dupont NEN; Boston, MA). The slides were incubated for 60 min at 23°C in phosphate buffered saline (pH 7.4) containing 1 nM [^{3}H]-NMS with or without carbachol. Adjacent sections were used to determine areas of nonspecific binding by incubating in the presence of 1.0 μM atropine. All sections were given two 5-min rinses, dried and prepared for autoradiographic localization. The specific sites at which the [^{3}H]-NMS binding was displaced by carbachol represented the high affinity sites. The high affinity carbachol sites appeared to predominate in the ventral horn of the spinal cord, the hypoglossal nucleus, facial nucleus, superior colliculus, zona incerta, nucleus tractus diagonalis, septal nuclei, and lamina IV of the cerebral cortex (23). This is similar to the distribution of sites we found labeled with [^{3}H]-CD. For this experiment, sections were incubated for 2 hrs at 4°C in 10 mM sodium-potassium phosphate (8.1 mM Na_2HPO_4, 1.9 mM KH_2PO_4) buffer (pH 7.4) containing 200 mM sucrose and 5 nM [^{3}H]-CD (38.1 Ci/mmol, Dupont NEN; Boston, MA). Again, 1 μM atropine was used to determine nonspecific binding. The sections were all given three 1 sec rinses in fresh buffer at 4°C before drying and autoradiographic localization.

II. Localization and Quantitation of Muscarinic Receptor Transport.

Standards (known amounts of tissue containing a predetermined amount of radioactivity) were developed so that the autoradiographic grain densities overlying labeled tissue sections could be quantitated. These standards are made of sections of homogenized animal brain in which a known amount of radioactivity has been added. The paste is frozen into a cylinder from which 10 micron thick sections are cut in a cryostat and thaw-mounted onto glass microscope slides. Our standards consist of 11 such sections which contain radioactivity ([^{3}H]leucine, specific activity 51.0 Ci/mmol, ICI Corp.; Philadelphia, PA) varying from a few hundred to several thousand counts per minute per section. By including these standards on the exposure of each sheet of autoradiographic film, it is possible to generate specific grain densities which can be associated with a known concentration of radioactivity. A standard curve can thus be produced which allows the grain densities appearing over various tissue regions to be matched with calculated concentrations of radioactivity from the standards. By knowing the specific activity of the radioactivity present in the tissue region and the efficiency of the counting system, the femtomoles of ligand bound in each area can be determined. By incubating serial sets of sections in various concentrations of radioactive PZ and/or QNB, it is possible to generate a saturation curve from which Scatchard analysis allows the identification of the B_{max} (number of receptors) and the K_D (a measure of affinity) for each microscopic region of the brain.

We are performing this type of analysis on the transported ligand-binding sites in the fimbria of the central nervous system and in the vagus and sciatic nerves of the peripheral nervous system. These lesions were made by electrolytic lesion of the fimbria or placement of a silk ligature on the sciatic or vagus nerve 24 hrs before sacrificing the animals. Longitudinal sections were obtained through the lesion site in preparation for autoradiographic localization. Our preliminary results indicate that neither subtype of muscarinic cholinergic receptor is solely presynaptic; rather, both muscarinic cholinergic receptor subtypes appear to be located on the pre- and post-synaptic membrane components of the cholinergic system (7).

III. Nicotinic Receptors.

We have attempted to use [^{3}H]-nicotine (specific activity 71.2 Ci/mmol, Dupont NEN; Boston, MA) to label nicotinic cholinergic receptors on slide mounted tissue sections of brain prior to autoradiographic localization of these sites. Our initial biochemical experiments indicated that we could obtain specific labeling of the nicotinic receptor by preincubating sections for 15 minutes in 50 mM Hepes buffer containing 1.2 mM $MgSO_4$, 118 mM NaCl, 25 mM $CaCl_2$, and 4.8 mM KCl (pH 7.4) at 25°C. This preincubation was followed with a 90 minute incubation in the same buffer containing 50 nM L-[^{3}H]-nicotine. The incubations were terminated by a 20 second rinse in fresh buffer without added radioactivity.

Specific binding (that displaceable with 1 mM concentrations of either tubocurarine, hexamethonium, or unlabeled L-nicotine) was detected in the interpeduncular nucleus, medial habenula, superficial layer of the superior colliculus, ventral posterior nucleus of the thalamus, and lamina IV of the parietal cortex. Less dense binding was present in the dorsal nucleus of the lateral geniculate body, lateral thalamic nucleus, medial geniculate body, accessory optic nucleus and dorsal tegmental nucleus. Only low levels of binding were found in the subiculum and molecular layer of the dentate gyrus in the hippocampal formation.

IV. Localization and Quantitation of Choline Uptake Sites.

Choline uptake sites can be labeled, as a marker for cholinergic nerve terminals, with [^{3}H]HC-3 (specific activity 120 Ci/mmol, Dupont NEN; Boston, MA). To accomplish this selective labeling, slide-mounted tissue sections were preincubated for 20 minutes at 25°C in phosphate-buffered saline (pH 7.4) containing 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 20.3 mM NaH_2PO_4 and 10 mM D-glucose. The sections were transferred to fresh buffer containing 2.5 nM [^{3}H]hemicholinium-3 for a 30 minute incubation period. This was terminated by rinsing the slides for 2 minutes in fresh buffer at 4°C. Areas of nonspecific binding were determined by including 1 µM unlabeled hemicholinium-3 in the incubation media of adjacent sections. This technology provides a novel and unique method for localization and quantitation of cholinergic nerve terminals in microscopic regions of the brain. We are currently examining the distribution of these sites and attempting to correlate the results with regions of known cholinergic innervation.

IV. Identification of Peptide Neuromodulatory Sites Associated with Cholinergic Function.

We have successfully defined the conditions for labeling receptors for two peptides (somatostatin and TRH) which are thought to be involved in the modulation of cholinergic nerve function. Labeling of the somatostatin receptors can be accomplished using a [^{125}I] labeled somatostatin analog as we recently described in collaboration with Dr. Henry Yamamura. Slide-mounted tissue sections were incubated for 60 min at 25°C in 50 mM Tris HCl buffer (pH 7.4) containing 20 µg/ml bacitracin, 2 mg/ml bovine serum albumin, 5 mM $MgSO_4$ and 0.8 nM [^{125}I]des-Ala^1-,Gly^2-desamino-Cys^{3}Tyr^{11}-dicarba^{3,14}-somatostatin (CGP23,996, specific activity 748 Ci/mmol, Ciba-Geigy Corp.; Summit, NJ). The sections were given two 20 minute rinses in fresh buffer at 4°C without added radioactivity.

Nonspecific binding was determined by incubating sections in the presence of 1 μM unlabeled somatostatin. To obtain labeling of the TRH receptors, slide-mounted tissue sections were incubated for 6 hours at 4°C in 20 mM sodium phosphate buffer (pH 7.5) containing 2 nM [^{3}H]-[3-Me-His^2] TRH (specific activity 70.4 Ci/mmol, Dupont NEN; Boston, MA). The incubation was followed by two 5 minute rinses in fresh buffer. Nonspecific binding was determined by incubating serial sections in the presence of 10 μM unlabeled TRH.

VI. Second Messengers.

Second messenger systems also involved in cholinergic nerve function can be localized and quantitated by autoradiographic techniques. Slide-mounted tissue sections can be treated with [^{3}H]forskolin (a diterpene plant derivative which is a potent activator of adenylate cyclase) to label adenylate cyclase. We are currently defining the conditions for labeling this enzyme and should be able to provide the first localization of these sites using quantitative techniques of receptor autoradiography. We have also very recently outlined preliminary conditions for obtaining labeling of guanine nucleotide binding proteins with [^{125}I]gamma-thio-GTP.

Discussion and Conclusions

We have established the conditions appropriate for obtaining selective labeling of muscarinic cholinergic receptor subtypes in the brain. Differential labeling of these sites (M_1 and M_2) show they are independently distributed in many brain regions and thus probably subserve different functions. These studies also include the localization of high and low affinity muscarinic receptors on the basis of agonist (carbachol) displacement of antagonist ([^{3}H]-NMS) binding. Only a few regions showed selective displacement by carbachol indicating structures with predominantly high affinity sites. These are the structures which were not labeled with [^{3}H]-PZ indicating that the M_2 receptor subtype predominates in these areas. It appears that agonist displacement of [^{3}H]-NMS binding preferentially reveals the high affinity M_2 receptor population. We recently verified this conclusion by demonstrating the guanine nucleotide sensitivity of these carbachol-displaceable [^{3}H]-NMS binding sites. The high and low affinity states of the M_2 cholinergic receptors are thought to be interconvertible by treatment with guanine nucleotides (24). The M_1 receptor agonist states may exhibit the same property with regard to the compound N-ethylmaleimide (NEM), although this has not been adequately demonstrated (24). Incubation of tissue sections in the presence of [^{3}H]-NMS and 100 μM concentrations of the non-hydrolyzable GTP analog Gpp(NH)p converts the high affinity carbachol agonist sites to their low affinity conformation. Sections incubated in this manner were compared with others incubated under the same conditions but in the absence of the guanine nucleotide (Gehlert and Wamsley, in preparation). The guanine nucleotide was shown to convert the high affinity M_2 receptor to the low affinity state, thus reducing the carbachol displaceability. These sections appeared similar to those labeled with the tritiated antagonist without any agonist present.

It is possible to directly label the high affinity sites with the agonist [^{3}H]-CD. Again, these sites showed a distribution similar to that expected from previous studies performed with agonist displacement of [^{3}H]-NMS. There were, however, a few notable exceptions which indicated

that certain ligands may preferentially label the high affinity M_1 receptor subtype in some regions of the brain and spinal cord. As a result of the binding studies described in this section, we are prepared now to examine adequately these muscarinic cholinergic receptor subtypes in animals treated with single and repeated doses of anticholinesterase agents. The development of the quantitation technology will also be valuable in analyzing the effects of acute and chronic exposure to anticholinesterase agents on either the M_1 or M_2 receptor populations.

Assessment of the density and distribution of nicotinic cholinergic receptors in the brain is now possible using quantitative autoradiographic techniques. We were able to show specific binding sites for [^{3}H]-nicotine and identify the individual nuclei of the brain which display these sites. The presence of nicotinic cholinergic receptors in many of these areas was previously demonstrated using an iodinated form of the snake venom alpha-bungarotoxin (25). This nicotinic cholinergic antagonist was shown to be specific for labeling nicotinic sites at the neuromuscular junction. Its efficacy in labeling the nicotinic sites in the central nervous system, however, was questionable, since it was not always possible to block cholinergic function in the presence of alpha-bungarotoxin at the nicotinic cholinergic synapse (26). Since alpha-bungarotoxin is an antagonist, our study with the nicotinic agonist L-[^{3}H]-nicotine may label only that high affinity agonist sites of these receptors since the distributions appear to be different in several areas. Thus, we suspect that only a subpopulation of the total nicotinic cholinergic receptors are labeled with L-[^{3}H]-nicotine (since an agonist should preferentially label high affinity sites, if they exist). This cannot account for the pattern of labeling identified in areas such as the cerebral cortex. In that structure, much of the labeling of alpha-bungarotoxin was found in several laminae, excluding lamina IV of the cerebral cortex (27). L-[^{3}H]-nicotine binding, on the other hand, was found solely in lamina IV of the cerebral cortex. Thus, at least in this structure, the labeling of L-[^{3}H]-nicotine is represented by binding to receptors not previously shown to be labeled with alpha-bungarotoxin (which should recognize all receptor subtypes with equal high affinity).

My laboratory, having developed the [^{3}H]-nicotine binding assay, is thus in a position to analyze the brain nicotinic cholinergic receptors and to compare the effects of anticholinesterase exposure on the distribution and density of these receptors with those of muscarinic cholinergic receptor populations of the brain. Correlation of cholinergic innervation with the density of muscarinic or nicotinic receptors would be of great value. This would provide a method for comparing damage to cholinergic neurons by anticholinesterase exposure with alteration in cholinergic receptor subtypes. It is now possible to quantitate the density of cholinergic nerve terminals using [^{3}H]HC-3 to label high affinity choline uptake sites and therefore provide a means of assessing damage to these terminals inflicted by anticholinesterases.

We originally proposed, as a means of analyzing the effects of anticholinesterase exposure on the density of cholinergic nerve terminals, that cholinergic neurons would be identified by cholinesterase staining. This technique is less sensitive than staining by the use of monoclonal antibodies directed to the cholinergic synthetic enzyme choline acetyltransferase. The latter technique, however, involves enzymatic activity associated with various parts of the neuron and would not be specifically directed towards the localization of terminals. Thus, the

localization of cholinergic terminals with hemicholinium-3 represents the latest advance in methodology designed to determine the density and distribution of these terminals in specific brain areas. This technique will allow us to define the effect of acute and chronic anticholinesterase exposure on these cholinergic nerve terminals.

Finally, we have utilized receptor autoradiographic techniques to identify peptide receptors associated with cholinergic nerve function. Both somatostatin and TRH receptors have been identified and their distributions are currently under investigation. These labeling parameters will be useful in ascertaining the effects of chronic and acute anticholinesterase exposure on neuropeptide receptors possibly associated with the regulation of cholinergic nerve function.

In addition to these receptor studies, it may soon be possible for us to analyze second messenger systems as well. We can selectively label both adenylate cyclase and guanine nucleotide binding proteins for localization and quantitation. Both of these substances appear to be concentrated in cholinergic and catecholaminergic terminals. It will be important to establish how the enzymatic and binding protein activities are altered in response to the chronic presence of anticholinesterase agents.

Significant progress and improvement has been made in our ability to assess the receptor and enzymatic effects of chronic exposure to anticholinesterases. The proposed analysis of these parameters following soman administration will soon be possible.

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