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13. ABSTRACT *(Maximum 200 words)*

Studies were conducted on the induction, expression, and stabilization of long-term potentiation (LTP), a form of synaptic plasticity that is likely to participate in memory encoding. Induction was shown to involve a glycine receptor site that modulates calcium fluxes through a subclass of transmitter receptors. Other results indicated that LTP expression is not likely to involve release or changes in spine increased resistance, but did provide direct evidence that potentiation reflects a change in the conductance properties of post-synaptic receptors. The hypothesis was developed that stabilization of LTP involves a disconnection and reconnection of adhesive relationships that maintain the organization of the synaptic region. This involves a calcium sensitive protease that cleaves cytoskeletal proteins and the exposure of a group of adhesion receptors known as integrins. Together with results from previous years of support, and from other laboratories, work over the past year has led to a reasonably complete hypothesis concerning how synapses can be rapidly transformed from one stable state to another and thus be used as memory storage devices.

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PROGRESS REPORT

1. INDUCTION OF LTP

Work in other laboratories found that the NMDA receptor, which figures prominently in the triggering of LTP, is modulated by an associated glycine site. Studies in our laboratory established that quinoxaline derivatives block this glycine site (Kessler *et al.*, 1989); similar observations were made at about the same time by another group who also identified 7-chlorokynurenic acid (7-CK) as being relatively selective in this regard. Work here showed *i)* that 7-CK is highly effective in preventing the induction of LTP (Oliver *et al.*, 1990a,b; copy attached), and *ii)* in collaboration with K. Baimbridge, that the glycine site modulates the influx of calcium mediated by the NMDA receptor (Oliver *et al.*, 1990c). Earlier studies in our group (Lynch *et al.*, 1983) and since replicated and extended by other investigators (Malenka *et al.*, 1988) indicate that changes in post-synaptic calcium are an essential step in producing LTP.

2. EXPRESSION OF LTP

The expression of LTP is usually discussed in terms of increased release, decreases in spine resistance or changes in receptors. Work supported by AFOSR grant #89-0383 argues strongly against release and resistance, and has provided direct evidence that modifications in the AMPA subclass of glutamate receptors are responsible for LTP expression.

a) Evidence that LTP is not due to increased transmitter release

Two groups of studies were carried out to test the release hypothesis. First, several manipulations known to increase or decrease release (calcium concentration, paired pulse facilitation, phorbol esters) were tested for differential effects on control vs. potentiated synapses in the same slices. In all cases, the treatments produced the same percentage effects on the two inputs (Muller and Lynch, 1989a; Muller *et al.*, 1990). If LTP were adding to the same variable as the manipulation, then the manipulation would not be expected to have the same percentage effects on the potentiated pathway (e.g., two treatments that increase release do not have multiplicative effects; see Muller and Lynch, 1989b for a demonstration in hippocampus). Second, if LTP was due to an increase in release, then it would be expected to enhance the responses generated by both AMPA and NMDA subclasses of glutamate receptors; if expression was due to a selective post-synaptic change (e.g., receptor modifications) then a disproportionate effect on the two classes of receptors would be expected. Three paradigms were developed to test these predictions and, as described in previous Progress Reports, it was found that LTP increased AMPA receptor mediated responses with little change in EPSPs generated by the NMDA receptors (see Muller and Lynch, 1988b; Muller *et al.*, 1988b; Muller *et al.*, 1989).

b) Evidence that LTP is not due to a decrease in spine resistance

Post-synaptic effects that could express LTP include receptor modifications and changes in spine biophysical properties such that a greater amount of synaptic current reaches the parent dendrite. With regard to the latter idea, it might seem that a reduction in spine resistance should facilitate the flow of currents produced by both classes of glutamate receptor and, as noted, this does not occur. However, computer simulations indicate that decreased neck resistance would have much larger effects on fast than on slow synaptic responses (cf. Wilson, 1984, and references therein) and it is the case that AMPA currents are considerably faster than those through NMDA receptors. The simulations assume a high neck resistance which governs the voltage drop at the spine head produced by fast synaptic currents. This voltage drop reduces the driving force for the synaptic current and hence reduces the current itself, in essence increasing any non-linearities between conductance and current. Decreasing the neck resistance would then serve to reduce the voltage change across the synapse elicited by a given conductance and thereby augment the current produced by that conductance. The voltage changes produced by slow synaptic currents could, according to theoretical considerations, be regulated ("clamped") by dendritic capacitance and hence be much less influenced by longitudinal spine resistance. Thus, it is not implausible that a change in spine biophysical properties would selectively amplify AMPA vs. NMDA receptor dependent responses.

Two tests of the spine resistance hypothesis were carried out during the tenure of the AFOSR grant. First, the hypothesis predicts that experimental manipulations that greatly reduce synaptic conductance should have greater effects on potentiated than control responses. This was not confirmed, specifically, blockade of as much as two-thirds of the post-synaptic response with antagonists of the AMPA receptors reduced potentiated and control potentials to an equivalent degree (Jung *et al.*, 1990a); this would not be expected if the non-linear curves relating synaptic current to synaptic conductance were markedly different for the two cases. Second, slowing the AMPA currents with cooling until they had the same time course as NMDA currents did not block LTP (Larson and Lynch, 1991).

c) Evidence that LTP reflects changes in glutamate receptors

As mentioned, above, LTP is expressed by an increase in synaptic currents generated by the AMPA subclass of glutamate receptors with much smaller effects on those mediated by the NMDA subtype. Perhaps the simplest argument that satisfies this constraint is that LTP involves a selective modification of AMPA receptor properties. Evidence that this idea is plausible comes from our studies (Terramani *et al.*, 1988; Massicotte *et al.*, 1990) and elsewhere showing that the ligand binding and ionic conductance characteristics of the AMPA receptor are selectively affected by a variety of manipulations. However, while it is clear that the AMPA receptor can have different conformation states that affect its binding properties, attempts to demonstrate that induction of LTP increases binding have not been successful. High frequency stimulation was administered at high voltages to some thirty sites in the field CA1 and the appropriate synaptic target regions processed for biochemical or autoradiographic analyses of AMPA and NMDA binding. No significant changes from control stimulated slices were found (see Lynch *et al.*, 1990; a much more extensive experiment with similar results is being prepared for publication). These negative results could indicate that LTP does not change receptor affinities or numbers or that these changes do not persist through the procedures involved in assaying binding characteristics.

Other AFOSR supported work in our laboratory has resulted in direct evidence that AMPA receptor changes are responsible for LTP. As noted earlier, if LTP represents an increment to one of the variables in the release-response equation, then drugs or other manipulations that add to the same variable should have proportionately smaller effects on potentiated synapses (see Staubli *et al.*, 1990c for a formal description of this point, copy appended). This result was not found in experiments using various treatments that affect the probability of release (see above) but it is obtained when using a drug that increases AMPA receptor conductance (Staubli *et al.*, 1990c). The nootropic compound aniracetam was found recently to enhance the conductance of AMPA receptors expressed in oocytes from rat brain mRNA without altering responses by NMDA and GABA receptors (Ito *et al.*, 1990). The same workers also showed that the drug increases the size of synaptic responses in hippocampus. Work in our laboratory using the two input paradigm indicates that aniracetam increases control responses by 25 ± 8% (n = 20) but potentiated inputs by only 14 ± 6% (n = 20; $p < .001$). Moreover, the drug has precisely the same percentage effects on responses transiently increased by a pre-synaptic manipulation (paired pulse facilitation) as it does in controls (see Staubli *et al.*, 1990c).

Subsequent experiments have replicated the above results and shown that aniracetam has no effect on NMDA receptor mediated responses (Xiao *et al.*, in prep.). These latter experiments used the paradigm established by Muller *et al.* (1988), namely low magnesium medium and the presence of the AMPA receptor antagonist CNQX; under these conditions field EPSPs are completely suppressed by NMDA receptor blockers and are unaffected by aniracetam.

It is also possible that the selectivity of aniracetam extends to the types or combinations of AMPA receptors expressed within a synaptic system. Work by Keinanen *et al.* (1990) indicates that three forms of AMPA receptors (Glu-A,B,C) each have a region in which one of two short amino acid sequences ("flip" vs. "flop") is inserted by the pertinent gene; these substitutes were shown to affect the conductance properties of the receptors. The dentate gyrus and CA1 express high levels of "flop" mRNA while field CA3 has high levels of "flip" (Sommer *et al.*, 1990). Preliminary studies indicate that aniracetam has a much smaller effect on the field EPSPs elicited by stimulation of the Schaffer-commissural system in the s. radiatum of field CA3 than in field CA1 (10 ± 5%; $\bar{X}$ ± SD, n = 6 vs. 27 ± 7%, n = 6). The drug had comparably large effects on the response of the dentate gyrus to stimulation of the perforant path (35 ± 10%, n = 11) as it did on the S-C responses in CA1. A simple

"flip" vs. "flop" explanation for these differences does not seem warranted given that Ito *et al.* (1990) found that aniracetam enhanced mossy fiber responses in CA3 to the same degree as S-C responses in CA1. If this last result is confirmed with further testing, it would seem likely that synapses in the distal apical dendrites of field CA3 contain a different combination of "flip" receptors than synapses in the proximal zones innervated by the mossy fibers. In any event, the regional variations in the effects of aniracetam indicate that the drug is extremely selective in its actions.

d) Summary

LTP has a much larger effect on AMPA receptor gated potentials than on NMDA receptor generated currents. A drug which increases AMPA receptor conductance produces smaller percentage effects on potentiated synapses. These observations, coupled with the negative findings discussed above, indicate that LTP expression is due to a change in the conductance properties of one class of glutamate receptors.

3. STABILIZATION OF LONG-TERM POTENTIATION

Other work supported by the AFOSR was directed at the hypothesis that the production of stable LTP involves a three step process: i) relaxation of the adhesive relationships that maintain the synapse, ii) a reconfiguration of the spine cytoskeleton and iii) re-establishment of junctional connections.

a) Time course for the production of stable LTP

In an attempt to gain an estimate of the time required for LTP to become stabilized slices were submitted to anoxic episodes (the O_2/CO_2 normally present in the atmosphere above the slices was replaced with N_2/CO_2) which were timed to begin at various intervals after high frequency stimulation. Anoxia depressed control and potentiated inputs to an equivalent degree; when this occurred within two minutes of LTP induction, the potentiated responses recovered to their pre-LTP baselines (LTP was reversed). Anoxic episodes beginning beyond the two minute period did not reverse LTP, even when prolonged to the point at which pathophysiology began to appear (Arai *et al.*, 1990). It thus appears that the machinery responsible for producing LTP can be disrupted for 1-2 minutes after high frequency stimulation after which the potentiation effect is extremely stable.

b) Pharmacological evidence implicating platelet activating factor (PAF) receptors in LTP

The brain is enriched in PAF receptors and is known to rapidly synthesize the trophic substance under some conditions. Since PAF triggers a large elevation in intracellular calcium in cultured hippocampal neurons (Kornecki and Ehrlich, 1988), and changes in intracellular calcium are necessary for LTP induction, PAF receptor activation is a potential contributor to the above described two minute development period. This idea was tested using an inhibitor (BTD; 2,5-bis(3,4,5-trimethoxyphenyl)-1,3-dioxolane) of the receptor. Since PAF levels are quite low in brain under resting conditions, it was not anticipated that blocking the receptor would have pronounced physiological effects. This was confirmed: BTD infusion for 40 min had no detectable effects on the slope, amplitude, or half-width of field EPSPs generated by stimulation of the Schaffer-commissural projections to field CA1. The drug, however, blocks the stabilization of long-term potentiation (del Cerro *et al.*, 1990a). This result has been confirmed using a second inhibitor of the PAF receptor and it has been found that an inactive version of the drug has no effects (Arai *et al.*, in prep).

c) Calcium activated proteases and LTP

The "disconnection-reconnection" hypothesis (Lynch *et al.*, 1991) requires that calcium stimulated cytoplasmic events disrupt adhesive relationships that maintain synapses in a stable configuration. Calcium activated proteases (calpain) could play an important role in this. The low threshold variant of these enzymes (calpain I) is activated by 1-5 μM calcium and thus while presumably inactive under resting conditions could be stimulated by the changes in calcium occurring after intense synaptic activity. Spectrin, a primary constituent of the membrane cytoskeleton and one that is concentrated in post-synaptic densities (Carlin *et al.*, 1983), is an excellent substrate for calpain (Siman *et al.*, 1984; Seubert *et al.*, 1987). Experiments by Harris and Morrow (1990) have shown that cleavage of spectrin by calpain releases the actin filaments normally held in place by the structural protein; this effect persists so long as calcium and calmodulin is present. Transmembrane membrane adhesion proteins belonging to the integrin family also attach to actin filaments via cross-linking proteins such as talin

and vinculin (Albelda and Bude, 1990). Thus, activation of calpain could create a transient situation (i.e., one lasting as long as calcium and calmodulin were present) in which integrin mediated cell-cell and cell-matrix connections were disrupted. Recent studies done in collaboration with U. Rutihauser suggest a second effect of calpain activation on adhesive relationships. These experiments used antibodies against various regions of N-CAM-180 and found that calpain I cleaves the cytoplasmic tail of this cell adhesion protein. The enzyme had no effect on N-CAM 120 (Sheppard *et al.*, 1991). N-CAM 180 is concentrated in post-synaptic densities (Persohn *et al.*, 1989) and very likely contributes to the stabilization of the synapse.

Two types of experiments have been conducted to test the hypothesis that activation of calpain is a necessary step in the production of stable LTP. The first of these asked if stimulation of NMDA receptors activates the protease and causes the cleavage of spectrin. As described in previous Progress Reports and Seubert *et al.* (1988) positive results were obtained.

A second group of studies tested if drugs known to inhibit calpain prevent the formation of stable LTP. The calcium requirement of calpain suggests that the protease should be inactive under normal circumstances and hence that blocking should not disturb baseline physiology. The study was done using *in vitro* slices; three hours of leupeptin infusion was sufficient to block LTP. The drug had no effect on baseline synaptic responses and importantly did not alter the responses occurring the high frequency bursts used to induce LTP (Oliver *et al.*, 1990c). Recently, a new calpain inhibitor (calpain inhibitor I, Boehringer) became available that is more potent and selective in its effects than leupeptin. This drug also blocked LTP and did so with shorter infusion times than required for leupeptin; it also did not alter the responses occurring during high frequency stimulation (del Cerro *et al.*, 1990b). The suppressive effects of the calpain inhibitors has been replicated by another group (Denny *et al.*, 1990; Soc. Neurosci. Abstract).

d) Integrin receptors and LTP

Studies were carried out with AFOSR support to explore the possibility that uncovering of integrin receptors participates in the stabilization of LTP. It is known that some integrins are normally quiescent and become functional only upon activation of the cell (Shimizu *et al.*, 1990). One subclass of integrin receptors, which includes members known to exist in different functional states, has a property which makes it possible to pharmacologically test for their participation in LTP. These receptors recognize the amino acid sequence arginine, glycine, aspartate (RGD) in their ligands and utilize this site for adhesion (Ruoslahti and Pierschbacher, 1987); moreover, the tetrapeptide RGDS partially blocks this interaction and has been shown to prevent a number of cases of cell adhesion. Infusion of RGDS into slices of hippocampus did not detectably affect any of several parameters of synaptic responses but did significantly reduce the degree of stable LTP that occurs after high frequency stimulation (Staubli *et al.*, 1990a. This effect has been replicated using GRGDSP and extended by showing that the blocking effect *i)* is fully reversible, and *ii)* is not reproduced by peptides lacking the RGD sequence (Bahr *et al.*, submitted).

The above results suggest that integrin-like proteins are present in synapses and participate in the stabilization of LTP. Efforts to identify such proteins have been made by applying solubilized proteins from synaptosomal membrane fractions to fibronectin columns and then testing for species displaced by RGDS. An RGD recognition protein doublet with molecular weights of 55 and 51 kDs (F-55) that is highly concentrated in synaptosomal membranes was obtained in this fashion. These polypeptides do not correspond in molecular weight to any known integrin subunit but they are recognized by an antibody to the $\alpha_5\beta_1$ integrin (the mammalian fibronectin receptor; Bahr *et al.*, submitted). Thus, integrin-like proteins are likely to be present in synapses and may play a role in the stabilization of LTP.

4. LIMBIC SEIZURES REDUCE LEVELS OF mRNA FOR A GLUTAMATE RECEPTOR SUBTYPE

Network models and theories have demonstrated the computational utility of mechanisms that reduce synaptic strength. These are usually hypothesized to be synapse specific and to become operative whenever pre- and post-synaptic activity are uncorrelated. Some models, however, postulate that the averaged activity of a cell affects its responsitivity to all inputs as well as its threshold for modification (see Bear *et al.*, 1989). Studies in collaboration with K. Sumikawa and C. Gall concerning mRNA encoding a subtype of AMPA receptor suggest a possible mechanism whereby the responsitivity of cells might be affected by activity.

These experiments became possible with the identification and sequencing by Heinemann and colleagues of a gene that encodes a functional AMPA receptor (Hollman *et al.*, 1990). Subsequent work by these investigators revealed that rat brain contains at least four homologous AMPA receptor genes and that the receptor proteins they encode can operate independently or in combinations to produce receptors with different properties. Using the sequence data published by Hollman *et al.* (1990), K. Sumikawa employed the polymerase chain reaction (PCR) to prepare cDNAs for the initially described AMPA receptor gene (Glu-A). Partial sequence analysis revealed that the c-DNA was identical to the sequence reported by Hollman *et al.* (1990). Electrophysiological studies using the oocyte expression system showed that cloned cDNA coded for a functional glutamate receptor. Three ^{35}S-labelled riboprobes were then prepared from linearized cDNA (Gall *et al.*, 1990). With these probes, it was possible to begin exploring the possibility that activity levels affect expression by the receptor gene.

Our first studies in this direction used the recurrent limbic seizure paradigm developed by C. Gall. With this, focal irritative lesions are placed unilaterally in the hilus of the dentate gyrus to produce bilateral epileptiform activity that spreads throughout the limbic system and neocortex. These episodes last for about a minute each and re-occur about five-ten times over an eight hour period. This technique provides a simple means for recurrently stimulating much of the forebrain. *In situ* hybridization analyses revealed that the levels of mRNA for the Glu-A receptor were dramatically reduced from 20-36 hours after the onset of seizure activity in dentate gyrus and superficial layers of cortex; smaller though reliable effects were also found in the hippocampal pyramidal cells. Normal levels of mRNA were found in all regions by 48 hours post seizure (see Gall *et al.*, 1990).

These results raise the possibility that activity levels in telencephalic circuitries occurring over days could affect the levels of AMPA receptors available for insertion into synapses. This would provide for a kind of reversible and generalized use-dependent plasticity upon which the synpase specific and stable LTP would be superimposed. It will be of interest to determine if repetitive electrical stimulation that does not cause epileptiform activity affects mRNA levels for AMPA receptor subtypes.

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