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The primary goal of the work conducted during the tenure of the AFOSR grant was to develop an adequate description of how environmental information is encoded into recognition memory by circuitries in mammalian forebrain. Most of the experimental effort was directed at identifying the cellular events responsible for the induction, development, and expression of long-term potentiation in the hippocampus, a stable form of synaptic facilitation that has been implicated in memory storage. Three major results were obtained: (1) A rhythmic pattern of physiological activity observed in hippocampus in animals engaged in learning is linked to the induction mechanism for LTP. The cellular events responsible for this linkage were identified and the first steps taken towards developing a set of rules describing the relationship between spatio-temporal activity and synaptic plasticity. (2) The expression of LTP involves an increase in the responses generated by one class of synaptic glutamate receptors with no changes in those produced by a second group; it can be concluded that one type of receptor induces LTP while a second type expresses it. This
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FINAL TECHNICAL REPORT

GARY LYNCH

A. Induction, Development, and Expression of LTP

1. A naturally occurring brain rhythm (theta) is linked to the cellular machinery that induces LTP.

2. Expression of LTP involves selective changes in responses produced by quisqualate receptors.

3. Evidence that a calcium activated protease (calpain) is critical to the development of LTP
   a) calpain and spectrin are found in dendritic spines
   b) NMDA receptors activate calpain
   c) inhibitors of calpain block LTP
   d) calpain produces morphological changes in blood-borne cells.

4. Role of protein kinase C in development of LTP

B. LTP and Memory: A Common Pharmacological Pathway

C. LTP and Cell Pathology

1. *In vivo* proteolysis of spectrin by calpain triggered with pathogenic manipulations

2. Calpain mediated spectrin proteolysis and ischemia

3. Spectrin proteolysis induced by kindling

D. Summary

The purpose of the proposed research was to test and elaborate on an hypothesis regarding cellular mechanisms responsible for storing recognition memory in mammalian telencephalon. We proposed that the encoding process involves: 1) an unusual pattern of physiological activity in the relevant neural pathways, 2) influx of calcium into dendritic spines postsynaptic to the active axons, 3) activation of the calcium-sensitive protease, calpain, 4) partial degradation of spectrin, a cytoskeleton protein that regulates membrane surface chemistry and possibly spine shape, and 5) anatomical reorganization of postsynaptic structure resulting in a stable increase of postsynaptic potentials. Central to the hypothesis is the phenomenon of long-term potentiation (LTP) of synaptic transmission; much of the research conducted in the past three years has been aimed at characterizing the physiological and biochemical steps responsible for this remarkably persistent synaptic change and examining its role in memory storage. We have also hypothesized that excessive activation of the calpain system can lead to the neuronal degeneration that is associated with experimental and age-related neuropathologies.

A. Induction, Development, and Expression of LTP

A.1. A naturally occurring brain rhythm (theta) is linked to the cellular machinery that induces LTP

For LTP to be a viable memory encoding mechanism, it should be produced by naturally-occurring patterns of cell discharges in memory-related brain structures such as hippocampus. We found that brief high frequency stimulation bursts, designed to mimic burst discharges of hippocampal neurons, induce optimal LTP when repeated at the period of the endogenous theta rhythm (Larson *et al.*, 1986). In chronic recording studies this pattern of stimulation produces LTP that persists without evident decay for weeks (Staubli and Lynch, 1987). The efficacy of this "theta burst" pattern derives from a period of IPSP suppression ("priming") that prolongs EPSPs and results in enhanced temporal summation of depolarization during a burst (Larson and Lynch, 1986). The enhanced depolarization allows postsynaptic NMDA receptor channels to be released from blockade by magnesium ions, thereby providing a route for calcium to enter postsynaptic cells (Larson and Lynch, 1988). Earlier work from our laboratory indicated that postsynaptic calcium triggers LTP (Lynch *et al.*, Nature 305: 719-721, 1983).

Further experiments have investigated LTP induced by asynchronous activation of synapses on a time scale relevant to a single theta cycle. When bursts were given in sequential but temporally overlapping order to three separate inputs to a collection of postsynaptic neurons, the degree of LTP induced was greatest in the first stimulated input, intermediate in the second, and least in the third (Larson and Lynch, 1989). These results imply that sustained depolarization after synaptic activation can maintain NMDA currents and facilitate LTP induction. Finally, when two inputs are stimulated in sequential but non-overlapping order, LTP is inhibited in the second set of synapses (Larson *et al.*, in prep). This suggests that LTP induction is followed by a brief refractory period, apparently due to the activation of a shunting conductance.

A.2. Expression of LTP involves selective changes in responses produced by one class of glutamate receptors.

One of the most unexpected and potentially important discoveries to have emerged during the tenure of the grant is that LTP affects only one of two major groups of glutamate receptors found in hippocampal synapses.

We made this observation while carrying out a series of studies designed to discriminate between pre- and post-synaptic substrates for the enduring synaptic potentiation, an issue that

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has been the subject of intense controversy. In the first group of experiments, we measured the effects of manipulations of transmitter release on expression of both LTP and paired-pulse facilitation (PPF). Hippocampal PPF was found to be virtually identical to neuromuscular PPF in terms of the effects of manipulations of extracellular calcium concentration, strongly suggesting that PPF in both systems is due to accumulation of calcium in presynaptic terminals (Muller and Lynch, 1989a). Induction of LTP had no detectable effect on PPF or its calcium dependency; however, 4-amino-piridine, an agent that increases presynaptic calcium currents and transmitter release, reduced PPF. These results suggest that LTP is not due to a potentiation of presynaptic calcium currents (Muller and Lynch, 1989b). The second series of experiments used several techniques for dissecting the contributions of non-NMDA and NMDA type glutamate receptors for responses evoked by transmitter release. Normally, NMDA receptors do not participate in transmission in hippocampus so we used five different manipulations to uncover them: (a) Under conditions of low extracellular magnesium (10-20 uM), primed single responses (i.e., responses tested 200 msec after stimulation of a separate input) are significantly reduced by the NMDA antagonist, AP5, (Muller and Lynch, 1988a). The response component evoked in the presence of AP5 represents the non-NMDA/quisqualate (QUIS) response; the component blocked by AP5 represents the NMDA component. Induction of LTP had a much larger effect on the QUIS component than the NMDA component; however, PPF resulted in marked facilitation of both components with the NMDA response being somewhat greater than the QUIS response (Muller and Lynch 1988b). (b) In physiological magnesium (1-2 mM), responses to a short high frequency burst under primed conditions (see above) contain a large D-AP5 sensitive (NMDA) component (Larson and Lynch, 1988). LTP greatly potentiated the D-AP5 resistant (QUIS) response but had little effect on the NMDA component (Muller *et al.*, 1989). (c) In low magnesium, two pathways in the same slice were used to examine the effects of previously induced LTP on "pure" NMDA receptor mediated responses. One pathway was potentiated by theta burst stimulation, the other served as a control. Antagonism of non-NMDA receptors with the drug DNQX reduced both potentiated and control responses to the same absolute size, indicating that NMDA receptors do not express already established LTP (Muller *et al.*, 1988a). (d) In the presence of low magnesium and DNQX, theta burst stimulation does not result in LTP of pure NMDA responses (Muller *et al.*, 1988a). (e) Washout of DNQX in these cases results in the expression of latent LTP on the previously stimulated pathway but not the control pathway (Muller *et al.*, 1988a).

These experiments provide compelling evidence that LTP is not expressed by NMDA receptors and contradict the idea that LTP is due to increased transmitter release. Three possible mechanisms could account for the observed effects: a) LTP is due to increased number or affinity of QUIS receptors, (b) a change in spine biophysics favoring fast QUIS responses over slow NMDA responses, and (c) new synapses lacking NMDA receptors.

A.3. Evidence that a calcium activated protease (calpain) is critical to the development of LTP.

A.3.a. Calpain and spectrin are found in dendritic spines.

The proposed role of cytoskeletal proteolysis in LTP and memory requires that both calpain and spectrin be present in dendritic spines. Immunocytochemistry at the EM level using a monoclonal antibody to calpain I confirmed the presence of calpain in dendrites and dendritic spines (Perlmutter *et al.*, 1988). Furthermore, subcellular fractionation experiments have shown that calpain I activity is highest in the soluble component of synaptosomal fractions (Baudry *et al.*, 1987). Polyclonal antibodies against brain spectrin have been used in several tests for calpain activation in vitro and in vivo (see below); these antibodies recognize both spectrin and breakdown products produced by calpain. We have shown that these antibodies label dendrites and dendritic spines in brain (Ivy *et al.*, 1988b). A contrary report (Reiderer *et al.*, J. Cell G

Biol. 102: 2088-2097, 1987) did not control for washout of spectrin from unfixed tissue during histochemical processing.

A.3.b. NMDA receptors activate calpain

Strong tests of the role of calpain/spectrin in LTP will require demonstration of spectrin proteolysis associated with LTP induction. A major problem in studies of this type is maximizing the population of synapses exhibiting potentiation in the tissue subjected to biochemical assay. Electrical stimulation activates an unknown and probably small fraction of synapses within a hippocampal slice and hence the ratio of potentiated to unaffected synapses is likely to be small. To circumvent this problem, we investigated the possibility that LTP might be induced by pharmacological means under conditions (bath application of drugs) where substantial populations of synapses are affected. We developed a technique in which application of NMDA reliably induced a synaptic potentiation effect that is very similar to electrically induced LTP (Thibault *et al.*, 1989). Slices were perfused in low magnesium medium (20 uM) to facilitate NMDA responses; brief (3 sec) application of NMDA in conjunction with glycine (an NMDA channel stimulant), spermine (a polyamine that alters mitochondrial calcium regulation) and high calcium was used to induce potentiation. Potentiation was antagonized by D-AP5 and was not reproduced by the other treatments when NMDA was omitted. Using a somewhat different paradigm, we found that prior LTP induced by electrical stimulation reduced the degree of "chemical potentiation" and that prior chemical potentiation reduced the LTP induced by electrical stimulation (Joly *et al.*, in prep). When induced, chemical potentiation could remain stable for at least 1 hour. These results are encouraging in regard to tests for spectrin breakdown with synaptic potentiation.

In parallel with these studies, we examined the effect of NMDA on spectrin breakdown. Bath application of NMDA for 15 min results in a reversible depolarization of neurons. Immunoblot assay demonstrated that NMDA treatment greatly accelerated the production of the calpain-mediated spectrin breakdown product (BDP). Both the depolarization and spectrin breakdown were prevented by treatment with the NMDA antagonist, AP5. Neither NMDA treatment in the absence of extracellular calcium nor depolarization induced by KCl (50 mM) results in BDP formation (Seubert *et al.*, 1988b). Studies of spectrin breakdown using the chemical potentiation paradigm are currently in progress.

A.3.c. Inhibitors of calpain block LTP

We have completed two studies testing the effects of the calpain inhibitor, leupeptin, on LTP induction. In the first, the drug (20 mg/ml) was chronically infused into the lateral ventricles of rats prepared with chronic stimulation and recording electrodes in the CA1 field. Leupeptin infusion had very little effect on baseline evoked responses but when LTP was tested (3-5 days after beginning infusion) only 3 of 13 animals showed potentiation one day later and only one exhibited stable potentiation for several days. Control animals with saline pumps all (11 of 11) exhibited robust and stable LTP. The block of LTP by leupeptin was reversible: after disconnection of the pumps, LTP was induced in 6 of 7 cases (Staubli *et al.*, 1988).

In the second study, hippocampal slices were incubated in the presence of 40-100 uM leupeptin. Incubation for 2 hr or less with the drug had no effect on LTP but after 3 hrs or more, LTP was significantly reduced (Oliver *et al.*, 1989). Examination of the postsynaptic responses to the theta burst stimulation used to induced LTP indicated that leupeptin had no significant effects on the depolarization and presumably the NMDA response in the slices in which LTP was reduced. Studies using recently developed and more selective calpain inhibitors are presently in progress.

A.3.d. Shape change induced by calpain-spectrin interactions

The hypothesis outlined at the beginning of this report requires that the calpain-spectrin interaction produce the structural changes that we and others have observed to accompany LTP. While partial cleavage of the cytoskeleton underlying the membrane would a priori be expected to produce morphological reorganization, we have experimentally explored this point using two types of peripheral cells (platelets and red blood cells). Platelets transform from flattened smooth profiles to a globular shape with many protrusions upon treatment with certain agonists; using immunoblots, we and others have found that spectrin is degraded during shape change resulting in the appearance of the same BDP produced by calpain. Comparable results were found using erythrocyte shape change as the test system (Siman *et al* ., 1987). We have also found that calpain activation in both intact platelets and isolated platelet membranes leads to the expression of an increased number of fibrinogen receptors (Vanderklish *et al.*, in prep).

A.4. Role of protein kinase C in the development of LTP

Several investigators have reported that phorbol esters, activators of PKC, enhance synaptic transmission and have argued that activation of PKC produces LTP. We used several phorbol esters, differing in their lipid solubility, and found that the synaptic facilitation they induce is fully reversible on drug washout (Muller *et al.*, 1988b). The duration of facilitation was related to the lipid solubility and hence the washout time required. Further, the reported interference of phorbol ester induced facilitation with LTP appeared to be due to an interference of the drugs with the depolarization induced by high frequency stimulation (Muller *et al.*, 1988b). We also tested the effects of the PKC inhibitor, H7, which is reported to prevent both the induction and expression of LTP (Malinow *et al.*, Nature 335: 820-824). We observed robust and stable LTP induced by theta burst stimulation in the presence of H7 (75 uM) (Muller et al., 1988b). Furthermore, in our hands H7 application after LTP had been induced did not preferentially reduce potentiated vs. control responses, although other drug effects were observed (Arai *et al.*, in press). The most consistent effect of H7 in these experiments was a pronounced increase in cellular excitability that was associated with a loss of synaptic inhibition. Our results do not support the hypotheses that PKC activation is required for either induction or expression of LTP.

B. LTP and Memory: A Common Biochemical Pathway

If LTP is involved in encoding of certain forms of memory, it is expected that drugs which disrupt LTP should also interfere with memory storage. As noted, NMDA receptor antagonists potently block LTP and our results indicate that leupeptin also blocks LTP. We have previously shown that chronic intraventricular infusion of leupeptin disrupts memory storage in both a spatial (radial arm maze) and an olfactory discrimination task (Staubli *et al.*, Behav. Neur. Biol 40: 58-69, 1984; Brain Res. 337: 333-336, 1985). Leupeptin, however, did not affect acquisition of avoidance conditioning (Staubli *et al.*, Behav. Neur. Biol., 43: 287-297, 1985). We have also tested the effects of chronic infusion of the NMDA antagonist, AP5, on LTP in vivo and acquisition of a spatial task (water maze). AP5 blocked both LTP and behavioral acquisition but did not affect visual discrimination learning in the same apparatus (Morris *et al.*, Nature, 319: 774-776, 1986). We have now found that AP5 infusions also prevent the acquisition of olfactory discrimination problems without affecting retention of previously learned discriminations (Staubli *et al.*, 1989). These results suggest that certain forms of "data-driven" memory share a pharmacological commonality with LTP and also underscore the current conceptions of multiple memory forms.

C. LTP and Cell Pathology

C.1. In vivo proteolysis of calpain is triggered by pathogenic manipulations

Incubation of purified brain spectrin with purified calpain and calcium results in the appearance of a 150 kDa breakdown product (BDP) that is resistant to further proteolysis and is recognized by anti-spectrin antibodies. Production of the BDP is inhibited by the calpain inhibitor, leupeptin, and is accelerated three- to five-fold by calmodulin (Seubert *et al.*, 1987). We have refined an immunoblot assay for the spectrin BDP and can detect nanogram quantities of the peptide. We have used this assay to test for activation of calpain in vivo after various treatments: a) Lesion of the entorhinal input to the dentate gyrus results in a massive elevation of spectrin BDP which is indistinguishable from the BDP generated by the spectrin-calpain interaction in vitro (Seubert *et al.*, 1988a). The increase in BDP is evident within four hours post-lesion and is maximal at two days post-lesion when the BDP makes up 25% of the total spectrin immunoreactivity in the dentate. Traces of BDP are detectable for at least four weeks. Prior infusion of leupeptin suppresses the generation of BDP. The time course of BDP appearance, the observed increase in spectrin immunoreactivity (Ivy *et al.*, 1988a) and the known ultrastructural changes following lesion all suggest a significant amount of spectrin breakdown is occurring in dendrites. b) Injections of colchicine into hippocampus results in cell death particularly in dentate granule cells. This pathological response is preceded by an increase in spectrin breakdown which is leupeptin (but not chloroquine) sensitive and is predominantly occurring in the molecular (dendritic) layer (Seubert *et al.*, 1989a). c) In Brindled mice (a genetic mutant carrying a deficiency in copper metabolism) an increase in spectrin BDP appears throughout the forebrain (but not the cerebellum) at about postnatal day 12 (Seubert et al., in prep). Copper is an essential cofactor for several enzymes involved in oxidative metabolism. The increase in BDP likely reflects abnormal calcium buffering leading to calpain and thus activation. The reasons for the difference in BDP among brain regions may be due to several factors including differential susceptibility to lowered energy charge or the distribution/response of NMDA receptors (see below). d) Treatment of rats with trimethyltin, an environmental toxin, results in selective cell death in the dentate and CA1 fields of hippocampus. Massive spectrin breakdown accompanied this treatment (Turnbull *et al.*, in prep). Pre-treatment with an NMDA receptor channel antagonist reduces the extent of spectrin breakdown.

C.2. Calpain-mediated spectrin proteolysis and ischemia

Brief interruption of cerebral blood flow leads to the death of selectively vulnerable CA1 pyramidal cells several days later. We examined the effects of ischemia on spectrin proteolysis and found massive breakdown (35%) occurred during the terminal phase of CA1 cell death (Seubert *et al.*, 1989b). Events initiated during the ischemia which may be responsible for the demise of these cells are thought to include NMDA receptor activation, which suggested a possible early phase of proteolysis. Substantial spectrin breakdown (7%) was indeed found to occur within 15 minutes of the ischemic insult. Administration of an NMDA receptor channel antagonist (MK-801) prior to the ischemia, a treatment which largely preserves the CA1 cells, significantly reduced both phases of proteolysis. These data strengthen the link between NMDA receptor activation and calpain-mediated proteolysis. They further suggest that the inital burst of proteolysis may be a triggering mechanism in the predisposition of these cells towards delayed death.

C.3. Spectrin proteolysis induced by kindling

Kindling refers to the process whereby subconvulsive electrical stimulation of certain brain structures, when repeated daily, results in the progressive development of electroencephalo-

graphic and behavioral seizures. We kindled animals with amygdala stimulation (theta burst stimulation) daily until at least 5 motor seizures were evoked on consecutive days. Following the last convulsion, we dissected out several brain structures known to be affected by amygdala kindling and assayed them for spectrin breakdown in immunoblots. Control animals were given only low frequency (0.1 Hz) stimulation and did not develop seizures. Spectrin BDPs were increased by 53% in kindled brain structures; no proteolysis was observed in cerebellum, a structure not seizure-prone after kindling. These data indicate that intense physiological activity promotes the activation of calpain and spectrin proteolysis (Oliver *et al.*, unpublished data).

D. Summary

While very large questions remain unanswered, it is probably fair to say that the work over the past three years has brought us close to a coherent and reasonably complete account of how recognition memories are encoded into cortical networks. We now know that physiological activity patterns that occur during learning have a deep relationship with the cellular machinery that induces LTP via a surprising property of inhibitory synapses. As much as any other single observation, this result indicates that LTP is associated with memory encoding. It also provides a framework within which to develop LTP-based synaptic learning rules for neural network models.

As unlikely as it may seem, LTP is induced by one set of glutamate receptors and expressed by a second set. This result severely constrains hypotheses about the locus and nature of the stable alterations that are responsible; by far the most likely possibilities are alterations in the quisqualate receptor complex or in the shape and resistance of the dendritic spines. Previous electron microscopic work from our laboratory has identified morphological changes in spines as a correlate of LTP (Lee *et al.*, J. Neurophysiol. 44: 247-258, 1980). The events lying between induction and expression of LTP (i.e., the development process) continue to be the subject of considerable controversy but are likely to involve calpain. Work carried out over the past three years has established three critical lines of evidence pointing to this conclusion: 1) the enzyme and its substrate are located in the appropriate zones; 2) stimulation of NMDA receptors activates calpain; and 3) inhibitors of calpain block the development of LTP. In addition, work on simple peripheral cells has gone a long way towards establishing a firm link between calpain activation with spectrin breakdown and the occurrence of morphological changes.

Calpain cannot be the whole story if for no other reason than that the enzyme is regulated by a variety of intracellular devices. Moreover, the cytoskeletal changes unleashed by spectrin breakdown are presumably influenced by a number of enzymes. We first reported that inhibitors of calmodulin (and thus calcium-calmodulin kinases) block development of LTP (Finn *et al.*, Neurosci. Lett., 19: 103-108, 1978) and several other groups have replicated this. Protein kinase C has also been much discussed with regard to LTP but our work over the past three years failed to confirm essential pharmacological findings implicating the enzyme in the induction or expression of potentiation.

New evidence has also been obtained regarding the all-important question of the role of LTP in memory. Three quite different types of findings implicate the substrates of LTP in recognition memory in telencephalic, cortical networks: 1) rhythmic activity occurring during learning is related to induction (see above); 2) LTP appears as behavioral learning develops (Roman *et al.*, 1987); 3) drugs which block LTP selectively interfere with some forms of learning (see above). The behavioral paradigm (point 3) developed during the tenure of the

AFOSR grant allows us to compare the effects of drugs on encoding vs. recall, something that is of great utility in controlling for non-specific actions.

Finally, new observations have confirmed the hypothesis that the machinery responsible for inducing and developing LTP is capable of producing neuropathology. We have obtained direct evidence that toxins, denervation, genetic abnormalities, and ischemia trigger the calpain-spectrin interactions. Moreover, as the effect occurs well in advance of any morphological breakdown or physiological signs of cell deterioration, it is far more likely to be a cause rather than a consequence of pathology. This constitutes a strong argument for a plasticity-pathology continuum.

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