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The Biochemistry of Memory: A New and Specific Hypothesis

Gary Lynch and Michel Baudry

Summary. Recent studies have uncovered a synaptic process with properties required for an intermediate step in memory storage. Calcium rapidly and irreversibly increases the number of receptors for glutamate (a probable neurotransmitter) in forebrain synaptic membranes by activating a proteinase (calpain) that degrades fodrin, a spectrin-like protein. This process provides a means through which physiological activity could produce long-lasting changes in synaptic chemistry and ultrastructure. Since the process is only poorly represented in the brain stem, it is hypothesized to be responsible for those forms of memory localized in the telencephalon.

Despite intensive experimentation and theorizing, a satisfactory description of the biochemical processes that lead to information storage in the mammalian central nervous system has yet to emerge. Two fundamental conditions for an adequate hypothesis have not been satisfied. (i) The proposed cellular mechanism must produce functionally meaningful, extremely persistent neurobiological changes of a type that can account for the behavioral manifestations of memory. (ii) It must be amenable to selective manipulation. Both conditions—explanatory power and testability—impose major difficulties. Problems with hypothesis testing arise from the fact that memory, in both a physiological and behavioral sense, must be assumed to be a higher order phenomenon. So far as we know, the brain interacts with the environment through axon discharges and synaptic transmission and it follows that the substrates of memory are triggered by and act upon these physiological events. Put simply, the integrated activity of primary physiological and behavioral processes is a necessary but not sufficient condition for the occurrence of memory. Any manipulation of these processes will inevitably have an impact on memory but cannot reveal the nature of cellular events specific to it. For this reason, hypotheses that involve simple exaggeration of routine physiological events, no matter how correct they may be, will necessarily be difficult to experimentally verify or reject.

There is, of course, no a priori reason to assume that rarely used or highly selective chemical processes are involved in memory storage, but if not, then the analysis of the cellular events leading to mammalian memory is probably beyond present technologies. This leads to the question of whether the brain possesses chemical reactions that are not involved in its ordinary physiological functions but nonetheless are plausible intermediates in memory storage. By plausible we mean that the candidate process meets the conditions imposed by the behavioral properties of memory (that it accounts for memory). Thus the process would minimally have to (i) be induced by brief physiological events and (ii) produce a change in the operating characteristics of neuronal circuitries. A further condition required is that the change would last for a very long period (days, weeks).

In this article, we present a hypothesis about the biochemical processes involved in memory storage. The postulated mechanism is initiated by a signal that is unusual but not unlikely to occur and produces an irreversible change in a key component of synaptic chemistry. Moreover, because of its nature, it can be assumed that the process does not participate in the short-term operation of neuronal circuits. It may therefore be amenable to selective manipulation, and some preliminary behavioral data pertinent to this are described.

Assumptions About the Locus of the Memory Storage Mechanism

Certain limiting assumptions are commonly made about the locus of the substrates of memory. Both clinical and experimental evidence point to the conclusion that the hippocampus somehow participates in the formation or retrieval of memory (or both) (*1*) and that the cerebral cortex is associated with its storage (*2*). As to the participation of individual neurons, modification of the nucleus or of the main dendritic or axonal processes of the cell would result in the simultaneous change of countless circuits, thereby imposing severe limitations on the precision and amount of information that could be stored. Accordingly, most theorists have argued for a synaptic localization of the modifications that encode memory (*3*). A major problem with this conclusion has been the lack of a satisfactory demonstration that neuronal connections possess the requisite physiological plasticity. However, it is now known that very brief periods of intense activity in cortical and hippocampal pathways produce increases in postsynaptic potentials that can persist for months (*4*). This effect, long-term potentiation (LTP), so closely resembles the expected memory property of synapses—that is, it is triggered by brief physiological events (*5*), seems to be strengthened by repetition (*6*), and can persist indefinitely (*4*)—that its substrates have been suggested to be the same as those used in memory (*7*). This idea has received support from a demonstration that the amplitude of LTP is correlated with the speed of complex maze learning in rats (*6*). But whatever its role in behavior might be, the discovery of LTP removes one of the major obstacles to the assumption that the site of memory is to be found in synapses.

There are no a priori reasons to favor a pre- or postsynaptic locus for the hypothetical modification taking place at the memory synapses. However, the occurrence of LTP in monosynaptic hippocampal pathways provides an excellent opportunity to study the extent to which pre- and postsynaptic structures are capable of generating stable modifications after physiological activity. Various strategies have been used in this regard. Drugs that block certain classes of acidic amino acid receptors also inhibit synap-

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Table 1. Similarity of calcium effects on glutamate receptor binding and degradation of fodrin in rat synaptic membranes.

Criterion	Glutamate receptor binding	Degradation of fodrin
EC_{50} for Ca^{2+}, 30 μM	+	+
Mn^{2+}, Sr^{2+} substitutes for Ca^{2+}	+	+
Inhibited by leupeptin and *N*-ethylmaleimide	+	+
Time course of reaction (minutes)	5	5 to 15
Absent below 20°C	+	+
Absent in cerebellum	+	+

tic transmission in several hippocampal pathways (*8*). When high frequency stimulation is administered during the block, LTP is not found upon restoration of transmission (*9*). This result implies that interaction between transmitter and postsynaptic receptor is needed to trigger LTP, thereby implicating the postsynaptic side of the synapse in the generation of potentiation. Stimulation of large numbers of fibers, even if they arise in widely separated regions, produces a greater LTP than activation of smaller numbers of axons (*10*); since axo-axonic contacts are virtually never found in hippocampus, this result is best explained by assuming that the magnitude of LTP is related to the size of the postsynaptic events produced by high frequency stimulation. Perhaps the strongest support for a postsynaptic origin of LTP comes from experiments showing that injection of the calcium chelator EGTA into postsynaptic target cells prevents the development of LTP in those cells (*11*).

These experiments demonstrate that the postsynaptic aspect of hippocampal connections has the physiologically induced "plasticity" needed for memory. Accordingly, identification of the types of long-lasting effects produced by the LTP paradigm could serve to establish end points to the search for potential biochemical intermediates of memory.

High Frequency Afferent Stimulation Produces Postsynaptic Changes

A considerable body of experimental data suggests that an acidic amino acid, possibly glutamate or aspartate (or both), serves as the transmitter in several hippocampal pathways, including those exhibiting LTP (*12*), and this has led to an intense search for a corresponding amino acid receptor. A binding site for glutamic acid that exhibits most of the characteristics anticipated for this receptor has been identified. Briefly it has the same pharmacological profile as synaptic transmission in hippocampus (*13*), is located postsynaptically (*14*), and is highly purified in synaptic membrane fractions (*15*). Furthermore, agonists that are reasonably selective for the site have been shown to produce increased postsynaptic sodium fluxes in slices of hippocampus (*16*).

In light of these results, we attempted to measure the effects of brief high frequency synaptic activity on this site. In the initial experiments, stimulating electrodes were placed along a pathway known to exhibit LTP in slices of hippocampus, and large stimulation voltages were used to activate the greatest possible number of axons. Several slices were stimulated at high frequency in this fashion, after which they were pooled and membrane fractions prepared. We found an increase in binding of glutamate, and from Scatchard analyses it appeared that this was due to an increase in the number rather than the affinity of the receptors (*17*). While demonstrating that glutamate receptors exhibit some form of plasticity, this experiment was compromised by the excessive stimulation currents that were used. Accordingly, we repeated the study with slices of one hippocampal subfield ("minislices") and then, using multiple electrodes, stimulated a very large number of contiguous groups of axons with currents that produced typical postsynaptic responses. Moreover, by refining our biochemical procedures we were able to measure binding in single minislices, a step that permitted correlations between physiological and biochemical changes.

We again found an increase in glutamate binding and moreover were able to demonstrate that this was correlated with the induction of LTP. The effect lasted for 45 minutes, the longest time period used and was blocked by conditions that inhibited the development of LTP. Finally, the increase in binding was smaller or altogether absent in slices that did not exhibit LTP (*18*) (Fig. 1).

Electron microscopic studies of the hippocampus from rats in which LTP was induced have revealed a rounding of dendritic spines and an increase in the number of certain classes of synapses (*19*). These results, which are not obtained after repetitive stimulation at frequencies that do not produce LTP, have been replicated with the use of in vitro slices of hippocampus (*20, 21*) and can only be interpreted as indicating that high frequency synaptic activity causes significant changes in dendritic ultrastructure (*22*).

It now appears that LTP is a more complex phenomenon than was originally thought. Three forms of potentiation distinguishable by their half-lives (90 minutes, several days, indefinite) have been reported to follow the high frequency train (*6, 23*). The relation of the anatomical and receptor changes to these effects (as well as to each other) remains to be determined, but studies on the "kindling" effect suggest a link between LTP and changes in glutamate binding sites. Kindling is a persistent seizure-proneness brought about by repeated, widely spaced bursts of intense, high frequency electrical stimulation. Like LTP, kindling produces an increase in glutamate binding sites (*24*). If the uncovering of glutamate receptors is involved in LTP then we might expect that the capacity of the kindled hippocampus to exhibit the potentiation effect would be reduced or eliminated. Racine *et al.* (*23*) found that the short-lasting form of LTP was intact after kindling but that the more persistent type was not.

From this battery of results we can conclude that the postsynaptic face of the neuronal connections is quite plastic and can be substantially changed by physiological activity. This increases the likelihood that memory storage is postsynaptic. The above findings also offer the plausible neurobiological end point needed for the search for a biochemistry of memory; specifically, we can now ask what cellular processes are available to the synapse that produce rapid and long-lasting increases in receptors and changes in spine morphology.

Calcium-Activated Proteinases and Glutamate Receptor Regulation

Studies of LTP have also suggested a trigger for the hypothetical process that encodes memory. The potentiation effect is reduced in size and frequency of occurrence in slices maintained in low calcium medium (*25*) as is the above-described increase in glutamate binding (*18*). Moreover, as mentioned above, injections of the calcium chelator EGTA into target neurones block the induction of LTP without causing noticeable

changes in the size or stability of excitatory postsynaptic potentials (*11*). These results suggest that an influx of calcium triggers both LTP and the increase in glutamate binding that accompanies it; another role of calcium in LTP has been considered (*26*). With this in mind, we asked whether calcium, in concentrations that might be expected to occur after intense synaptic activity, changes the properties of sodium-independent glutamate binding to membranes isolated from hippocampus and other brain structures. We found that concentrations of calcium as low as 10 μM induce a substantial increase in the maximal number, but not the affinity, of glutamate binding sites in hippocampus, striatum, and cortex (*27*). Moreover, the calcium mediated increase in binding proved to be largely irreversible; thus extensive washing of the membranes with calcium-free medium or with EGTA after exposure to calcium did not eliminate the effect (*28*).

Having obtained these results, we set about to identify the underlying mechanisms. An important clue was the observation that the calcium effect was sensitive to temperature and was essentially absent at 20° to 25°C and reached a maximum at 35° to 40°C (*29*). This suggested that an enzyme or membrane modification (or both) was involved. Because the increased binding persisted after removal of calcium, it also seemed necessary to assume that transient activation of a calcium-dependent process produced stable consequences. This last and quite unusual property is reminiscent of the actions of proteinases since these enzymes, by causing the irreversible cleavage of peptide bonds, have effects that persist for an indefinite period after the enzyme itself has been inactivated. There is also a body of evidence linking proteolytic activity with the regulation of certain hormone receptors (*30*). Therefore, we began testing for possible influences of proteinases on glutamate receptors. We found that treatments that inhibit the activity of calcium-activated neutral thiol proteinases indeed blocked the increased binding produced by calcium (*31*). Perhaps the most convincing results were obtained with leupeptin, a tripeptide that selectively and potently inhibits this class of enzymes (*32*). This inhibitor has little or no effect on basal binding but thoroughly antagonizes the effects of calcium (*31*).

These findings provided strong, although still indirect, evidence that neuronal membranes contain a form of calcium-activated neutral proteinases—or calpain (*33*)—that regulates glutamate receptor binding. At the time that the results were obtained, proteinases of this type were known to occur throughout the body, but the enzyme was usually reported to require much higher calcium concentrations than were used in our studies. In the past 2 to 3 years, however, there have been studies indicating the existence of a variant of the enzyme (calpain I) that is activated by low micromolar concentrations of calcium (*34*). However, calpain is almost universally reported to be a soluble enzyme, while our hypothesis required a membrane-associated variant. To test this, we attempted to isolate calpain activity from synaptic plasma membrane fractions. Both low- and high-threshold calcium-stimulated proteinase activity could be extracted from rat brain synaptic membranes incubated in low ionic strength buffer. After partial purification by cellulose columns chromatography, the enzymatic activities were, by a large number of criteria, identical to the calpains purified from other tissues (*35*).

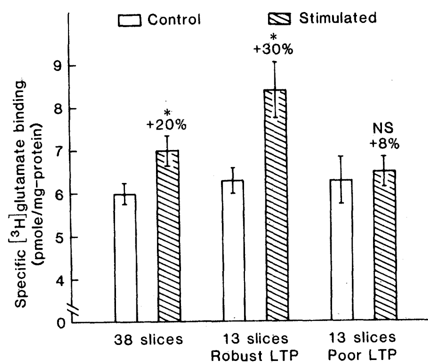

Fig. 1. Changes in ^{3}H-labeled glutamate binding to hippocampal membranes elicited by high-frequency stimulation in CAl "minislices". Fifteen minutes after high frequency stimulation to 24 to 40 sites on a CAl minislice, crude synaptic membranes were prepared and [^{3}H]glutamate binding was measured at a glutamate concentration of 100 nM. A control minislice was removed at the same time as the stimulated minislice. Data were collected from 38 stimulated and 38 control slices. Robust and poor LTP refer to the extent of synaptic facilitation produced in two subgroups (N = 13 each) of stimulated minislices. The control values are for the groups (N = 13 each) of slices removed and processed at the same time as the slices exhibiting robust or poor LTP. The asterisk indicates $P < 0.02$ t-test, two-tailed. The data are from (*18*).

Membrane Substrates for the Calcium-Activated Proteinase

The discovery of a membrane-associated calcium-dependent proteinase that uncovers glutamate receptors led to a search for its substrates. Membranes were incubated in the presence or absence of calcium, washed, and then dissolved, and the solubilized proteins were separated on polyacrylamide gels. Two sets of high molecular weight polypeptides were conspicuously reduced in the calcium-treated membranes. The larger set (320,000 daltons) appears to correspond to the microtubule associated proteins (MAP's), a known substrate of calcium proteinases (*36*). The apparent molecular weight of the second (doublet) peptide closely resembled that of two polypeptides (230,000 to 240,000 daltons) named α- and β-fodrin by Levine and Willard (*37*). The properties of the calcium-induced degradation of this protein closely resembled those for the uncovering of glutamate binding sites (Table 1).

The possibility that fodrin is a substrate for the membrane-associated proteinase was an important clue since this protein is known to line the inner face of neuronal membranes and is concentrated in postsynaptic densities (*38*). Moreover, an increasing body of data indicates that fodrin is functionally and structurally comparable to the erythrocyte membrane protein spectrin (*39*). Spectrin serves to link transmembrane proteins with the actin network on the inner face of the membrane and thus with the cell's cytoskeleton. The protein is widely held to regulate the mobility of surface receptors and possibly cell shape as well (*40*). For instance, both fodrin and spectrin participate in the capping of cell surface receptors in various cell types (*41*). If, as is not unlikely, fodrin plays a comparable role in neurons, then the local disruption of this protein might well be expected to produce the receptor modifications seen after incubation of membranes with calcium, or in slices exposed to high frequency synaptic activity. Disruption of the fodrin network could also account for the shape changes found in spines after the induction of LTP. With this in mind, we investigated the identity of the 23,000- to 240,000-dalton membrane polypeptide doublet that is degraded, in a leupeptin-sensitive fashion, by calcium. We found that fodrin, purified by the technique of Levine and Willard (*37*), comigrates with the high molecular weight doublet (*42*); moreover, purified brain fodrin is degraded by a purified calcium-dependent proteinase isolated from erythrocyte cytoplasm (*43*). Using labeled fodrin, we also found that fodrin and calpain have an affinity for each other in the nanomolar range, a value comparable to that for the other known substrates of the enzyme (*44*). These results leave little doubt that fodrin is an endogenous membrane substrate of calpain.

The Hypothesis

The above findings lead us to propose the following hypothesis about the way in which at least some central synapses achieve rapid, extremely long-lasting changes in their efficiency (Fig. 2). Brief bursts of high frequency activity cause a transient elevation of calcium in spines that activates a membrane-associated calpain. This enzyme then breaks up a localized portion of the fodrin network, producing structural and chemical changes in the region of the postsynaptic membrane. As a result, previously occluded glutamate receptors are exposed, thereby increasing the size of the postsynaptic response to the released transmitter. More prolonged or repetitive bursts of activity can be expected to produce a larger calcium disturbance and more widespread activation of the calcium-dependent proteinase, events that we propose will produce alterations in the ultrastructure of the dendritic spine (*45*).

Conceivably, a single burst of high frequency activity might do no more than uncover a population of receptors, but subsequent bursts acting on the now potentiated synapses would produce greater depolarization and hence greater calcium influx. Repetition then would (i) steadily increase the number of synaptic receptors and (ii) cause ever greater influxes of calcium and ultimately structural changes. Thus a type of practice effect, seen not only in behavioral memory but with LTP as well (*6*), would occur.

It is difficult to estimate the potential duration of these effects. Added receptors have half-lives perhaps of the order of several days. If so, the increase in the number of glutamate receptors might correspond to that form of long-term synaptic potentiation which has a half-life of 3 to 6 days (*21*). Alternatively, it is possible that the transient activation by calcium of calpain and the resulting degradation of the fodrin network induces the formation of new "hot spots" for the insertion of glutamate receptors in the postsynaptic membranes which could then remain for the life-span of the synapse. The life expectancy of structural changes depends on the factors that regulate the cytoskeleton and its linkages with the membrane; given that so little is known of this in neurons, it is perhaps best to say only that morphological alterations could last for very long periods.

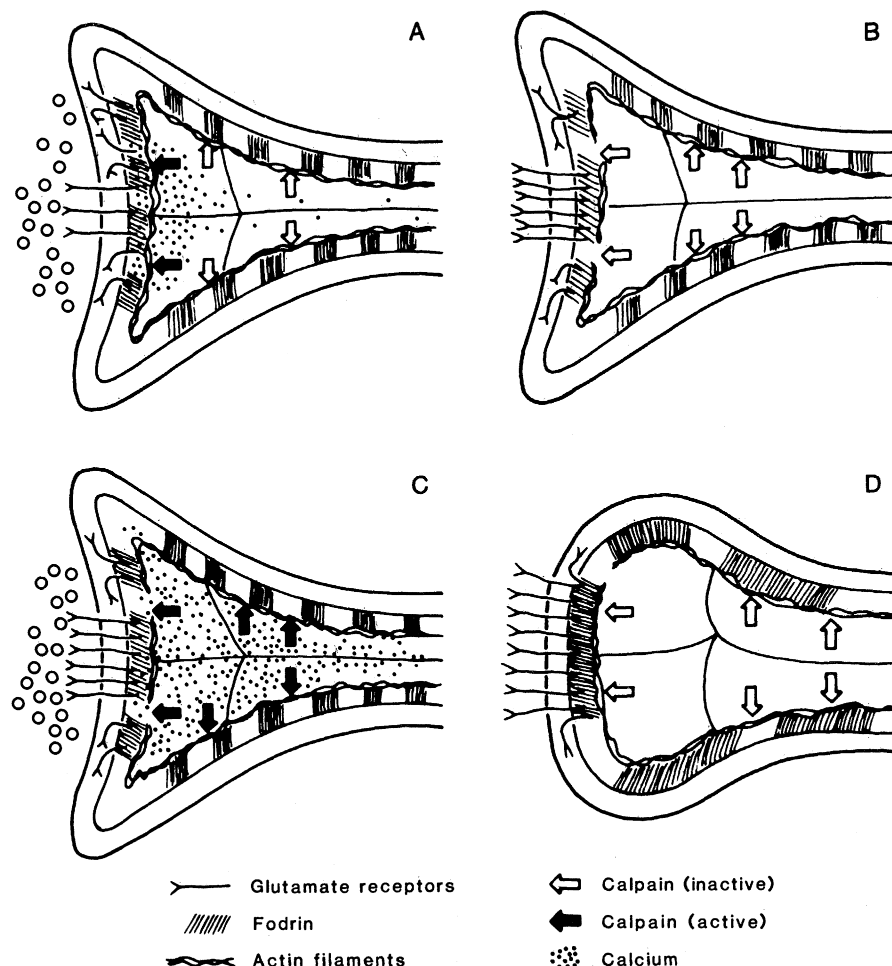

Fig. 2. Hypothesis concerning the mechanism by which brief periods of high frequency activity produce long-lasting changes in synaptic efficacy. (A) Transmitter release causes an increase in calcium in the subsynaptic zone activating calpain, which degrades fodrin and uncovers occluded glutamate receptors. (B) Calcium is removed from the spine inactivating the calpain. (C) Subsequent episodes of high frequency activity produce a larger influx of calcium because of the greater number of receptors. This stimulates calpain throughout the spine and leads to widespread disruption of the fodrin network permitting shape change to occur. (D) Calcium is again eliminated from the spine but the structural and receptor changes produced by transient activation of calpain remain.

Encephalization of the Proteinase-Receptor Interaction

It is now widely held that mammals, including humans, have at least two quite different forms of memory that utilize different brain structures for encoding or storage (*46*). Lesions of the hippocampus and related temporal cortical structures result in a severe impairment of tasks requiring spatial memory in rats but do not disrupt the learning of shock avoidance (*47*). In humans, hippocampal damage is associated with a profound disturbance of storage of new semantic information but leaves the patient with the ability to learn puzzles (*46*). Conversely, it has been repeatedly demonstrated that classical conditioning is readily obtained in decerebrate animals (*48*). Recent neurophysiological analyses have shown that conditioning of the eyeblink reflex involves brain stem circuitry and is abolished by very discrete lesions of the cerebellum (*49*).

In sum, there is good reason for believing that some forms of learning are mediated by lower brain structures while others involve the telencephalon. It does not follow necessarily that the biochemical mechanisms underlying these two forms of learning differ as well, but it is equally true that there is no reason, other than economy, to reject this possibility. Accordingly, we looked for evidence that the proteinase-receptor mechanism might vary across various brain structures. Calcium is about equivalently effective in uncovering glutamate receptors in cortex, striatum, and hippocampus but at low concentrations is virtually without effect in several brain stem regions (Fig. 3). The pharmacology and kinetics of the sodium-independent glutamate sites are indistinguishable across these regions (*14*). Thus some aspect of the proteinase-receptor mechanism is lacking in the brainstem; in confirmation of this we have found that calcium from 50 to 250 μM caused no detectable changes in either the α- or β-subunits of fodrin in cerebellum (*30*). Possibly cere-

bellar membranes lack the proteinase, or the enzyme does not have access to the fodrin and MAP proteins. There is indeed evidence for differences in the protein composition of synaptic membranes in cerebellum (*50*), and studies of the localization, properties, and substrates of membrane-associated calpain I from different brain regions as well as from nonnervous system tissue should reveal the nature of the differences.

From our experimental data described above it can be concluded that the postulated memory mechanism is operative only in telencephalic brain structures. Since these are the regions of brain most different in mammals compared with other vertebrates, the possibility exists that the mechanism is only slightly or not at all involved in learning and memory in nonmammals. There are other reasons for suspecting that this might be true. We have already noted that the calcium-induced increase in glutamate binding sites is severely reduced when assayed at room temperature (*28*). This would suggest that poikilothermic animals (amphibians and fishes) that inhabit temperate zones would not be able to utilize this process. Since these animals have a number of membrane and enzymatic adaptations to temperature, it is possible that the calpain-fodrin interaction occurs under conditions in which it is inoperative in mammals.

In our laboratory we have not been able to detect any evidence for calcium stimulation of glutamate binding in frog or newt brain membranes incubated with calcium at 20° or 25°C (*51*). Similar negative findings were obtained in reptile and bird brain membranes assayed at 30° or 35°C (*51*). It can be hypothesized that the regulation of glutamate receptors by calpain is not to be found in many vertebrates and indeed may be a mammalian invention. To summarize, the absence of the mechanism in cerebellum combined with our available data for amphibians and other vertebrates indicate that this mechanism cannot be responsible for simpler forms of memory. It may be that several forms of learning and memory exist, each mediated by different cellular processes, the modulation of transmitter release evidenced in invertebrates (*52*) being one.

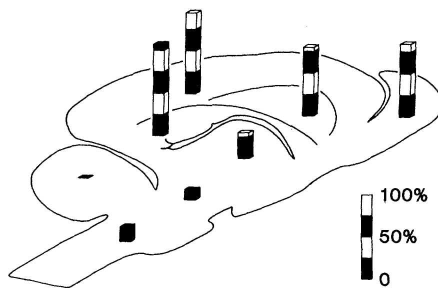

Fig. 3. Regional distribution of calcium stimulation of [^{3}H]glutamate binding in rat brain. The numbers represent the percent increase in [^{3}H]glutamate binding elicited by 50 μM calcium.

Behavioral Testing of the Hypothesis

The above-described hypothesis makes a number of testable predictions, prominent among which are the following. (i) Memory formation should be accompanied by an increase in glutamate receptor binding in telencephalic structures and (ii) drugs that inhibit the calcium-activated proteinase should block those forms of memory, and only those forms, that are dependent on telencephalic regions. The second of these renders the hypothesis subject to disproof (that is, memory occurs during inhibition of the enzyme).

Recent experiments have provided evidence pertinent to both predictions. Thompson and his associates have shown that conditioning of the eyeblink reflex in rabbits produces pronounced and widespread changes in the activity of hippocampal neurons (*53*) as well as a modification in the strength of monosynaptic evoked potentials (*54*). These physiological effects are not responsible for the behaviorally observed learning since this is unaffected by lesions of hippocampus—in fact, it appears that the cerebellar-brainstem circuitry is involved (*50*). Presumably the alterations in hippocampus are linked to learned behavior not sampled by the simple conditioning paradigms. Whatever their ultimate significance for learning, these observations afford an opportunity to ask whether long-lasting behaviorally induced changes in hippocampal physiology are accompanied by alterations in glutamate receptors.

A collaborative project of this type was undertaken with three groups of rabbits: (i) tone coupled with airpuff ("conditioning"), (ii) tone expressly not coupled with the airpuff, and (iii) naïve (that is, colony housed) controls. The rabbits were killed either at 1 or at 18 hours after the last of three daily sessions in the training apparatus, and assays of glutamate binding to hippocampal membranes were conducted "blind" (without knowledge of behavioral conditions). Binding was greater by 20 to 40 percent ($P < 0.01$) in the conditioned group than in the unpaired or control groups. Analysis of binding under equilibrium conditions indicated that the effect was due to an increase in the number of sites; there was also a tendency for the affinity of the site to change but this did not reach statistical significance (*55*). These results provide a clear demonstration that sodium-independent binding sites are uncovered by learning and thereby add support to the hypothesis that glutamate receptor plasticity is involved in memory storage. It is perhaps surprising that binding in the explicitly unpaired group was not different from that found in the naïve controls, since these animals must learn something about the training procedure (for example, that the tone is not a predictor of the airpuff). However, there is no necessary reason to assume that this learning involves hippocampus, and indeed Thompson and co-workers have shown that hippocampal physiology is not changed in rabbits exposed to random presentations of the conditioned stimulus and unconditioned stimulus (*53*). The absence of receptor changes in these animals suggests that the increase in receptors in the paired group was not due to stress induced by factors such as handling.

We have examined the effects on glutamate binding to hippocampal membranes of rat that have been exposed daily for 2 weeks to an enriched environment and have found increases comparable to those obtained in the rabbit conditioning studies (*56*). Therefore, we conclude that glutamate receptors are influenced by a variety of behaviors and that the rabbit is not an atypical species in this regard.

In order to test the prediction that inhibition of calpain will interfere with those forms of memory dependent upon telencephalic brain structures, we measured the effects of leupeptin on several behavioral indices (*57*). Osmotic minipumps containing one of three drug concentrations (4, 8, and 20 milligrams per milliliter of saline) were implanted in rats and connected to cannulae positioned into the lateral ventricle; these pumps operated continuously for 2 weeks and were calculated to produce leupeptin concentrations in the cerebrospinal fluid of 10 to 100 μM. Animals with implanted minipumps containing aprotinin, a potent inhibitor of serine proteinases, or saline were used as controls for any nonspecific effects of continuous intraventricular infusions. Leupeptin caused no detectable changes in food and water intake or in body temperature, and, with the exception of some animals in the high concentration group, did not influence spontaneous locomotor activity or rearing (Fig. 4). Two groups of memory tests were used. In the first, the animals were trained in a radial maze (*47*) prior to implantation of the pumps. In these

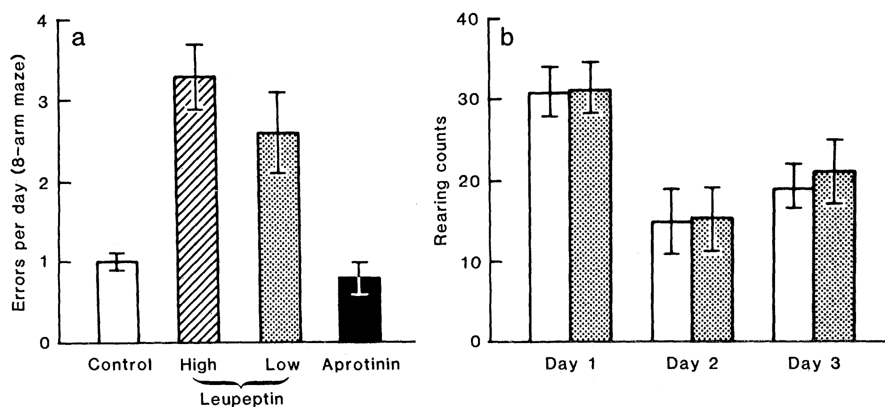

Fig. 4. Effects of proteinase inhibitors on various behavioral tasks. Osmotic minipumps (Alzet model 2002, Alza, Palo Alto, California) were implanted under the skin and connected by fine tubing to a cannula implanted into the lateral ventricle at the level of the anterior hippocampal commissures. The pumps (0.2 ml) were filled with saline (control), leupeptin, 4 mg/ml (leupeptin low), 20 mg/ml (leupeptin high), or aprotinin, 10 mg/ml (aprotinin). (a) Before the start of the drug phase of the experiment, the rats were trained for 2 weeks in the radial maze with one or two daily testing periods. Following this training period the osmotic pumps were implanted and 2 days later the rats were reintroduced to the maze. Results represent the means ± standard error of the mean of the median scores for each animal over the several days of testing. (b) Osmotic minipumps (leupeptin intermediate) were implanted into naïve rats. Three days later the animals were tested for rearing over a 6-minute period in a closed apparatus (32 by 32 by 36 cm). Results represent the means ± standard error of the mean of the rearing counts for three successive days of testing; data for animals infused with saline alone are indicated by the open bars, those for leupeptin (8 mg/ml) treated rats by stippled bars (*57*).

tests, the rats, maintained at 80 percent of body weight, were placed in the center of the maze and allowed to wander freely through eight outwardly extending arms at the end of each of which a small piece of chocolate is hidden in a recessed well. The animals quickly adopt an optional "foraging strategy" of not reentering arms (that is, of avoiding the arms from which they have already removed the chocolate). On some trials, the animals are removed from the center position between the fourth and fifth choices, placed in a holding cage for periods up to 4 hours, and then returned to the maze. Normal animals will choose the remaining four arms still containing the reward, using spatial cues in the area surrounding the maze to orient themselves (*47*). This task obviously involves a considerable memory component and, as shown by Olton and co-workers, is completely disrupted by lesions of hippocampus or its input-output pathways (*47*).

After the rats were tested for 10 to 14 days leupeptin-, aprotinin- or saline-containing pumps were implanted and connected and daily testing on the maze was resumed. Drug-treated rats quickly entered arms and consumed the reward, behaviors that are never seen in naïve animals, an indication that the maze was "remembered." However, both low and high concentrations of leupeptin produced serious impairments in the daily memory required for optimal performance (Fig. 4). Saline and aprotinin infusion had no apparent effect on maze behavior in any of the control rats.

Escape and avoidance learning were tested in an additional group of five drug- and five saline-treated rats. Leupeptin (high concentration) animals were indistinguishable from controls in learning to enter an adjacent chamber to escape from a mild footshock. The animals may have been slightly slower in learning to avoid (as opposed to escaping) the shock, but this difference did not reach statistical significance; in any event, each of the five leupeptin-treated rats successfully avoided shock at the end of a second day of eight trials. It is worth noting that hippocampal lesions do not disrupt this type of learning (*48*). Finally, leupeptin-treated rats were virtually identical to controls in their performance on a standard inhibitory avoidance problem. In more recent experiments, a very different type of behavioral problem was used to assess the effects of leupeptin on memories that involve telencephalic brain structures (*58*). Rats were trained on a series of different odor pairs to select one of two smells for a water reward. The positions of the odors were randomized on a trial by trial basis. After several pairs, the rats learn the correct odor in only two or three trials even when 10-minute delays are inserted between the trials. Leupeptin in intermediate concentrations greatly slowed the acquisition of the correct odor in well-trained rats (*58*). The projections of the olfactory system are for the most part restricted to the telencephalon and it is reasonable to assume that the changes responsible for the memory required in the successive discrimination task are localized to that portion of the brain.

These results confirm the prediction that inhibitors of the calcium-activated proteinase will disrupt those forms of learning that are critically dependent on the telencephalon. Whereas this considerably strengthens the hypothesis discussed above, the findings should not be overinterpreted. Leupeptin blocks thiol proteinases other than the calcium-activated enzymes (some lysosomal enzymes), and the behavioral significance of this is unknown. Moreover, there are no data on the relative degree of inhibition of these enzymes produced by intraventricular injections although we have found that brain calpain is significantly blocked under the conditions used in the behavior experiments. It is also a distinct possibility that leupeptin has as yet undetected side effects. However, the effects of the inhibitor were found at concentrations that did not produce any detectable changes in spontaneous activity, feeding, and drinking, and were not found when different types of simple escape and avoidance conditioning were included. Moreover, the drug has been tested extensively on muscle cells and has been reported to have no effects on protein synthesis and cytoskeletal integrity in normal cells (*59*).

Conclusion

Our conclusion is that the mammalian brain does possess a chemical mechanism that could account for memory and yet is not likely to be involved in the ongoing operation of neuronal circuitries. The calcium proteinase-receptor process matches the conditions imposed by the behavioral features of memory.

1) It is triggered by an event (increase in intracellular calcium) that can be expected to follow unusual physiological activity. In fact, this is known to occur after brief periods of high frequency activity (*60*).

2) It produces an effect (increased number of glutamate receptors) that should modify the functional properties of neuronal circuits.

3) Its consequences are extremely long-lasting.

4) It produces biochemical effects that are found after learning.

In addition, the process has several attributes that are highly desirable, if not

necessary, features of a memory mechanism. (i) It is localized to synapses. (ii) It appears to be restricted to telencephalic structures and may be limited to higher vertebrates. We can assume that the postulated memory mechanism does not participate in routine physiology simply because the activation of calpain requires calcium levels greater than can be expected to occur under normal conditions and indeed are in excess of concentrations needed for most calcium-mediated neuronal processes. Thus it should be possible to selectively manipulate the proposed biochemical intermediate mechanism and thus test the hypothesis that it produces memory.

Is the proteinase-receptor interaction responsible for a particular type of memory? The selective blockade of some forms of learning by the potent and relatively selective inhibitor leupeptin is strongly suggestive but, as discussed, the findings cannot be viewed as conclusive. It will be necessary to manipulate the enzyme by diverse means and test for common effects on memory. Endogenous inhibitors (*33*) and activators (*61*) of calpain have been described and further studies of these compounds can be expected to produce the pharmacologies needed for multifaceted behavioral tests. But perhaps the above question is wrongly phrased. If the activation is triggered by modest levels of calcium and produces irreversible changes in synaptic chemistry, then activation of the membrane-associated calpain would seem to be both a likely event and one that should produce lasting changes in the operation of neuronal circuitries. Although it is not logically necessary that such changes modify behavior, to conclude otherwise would require unusual assumptions about the manner in which the central nervous system operates.

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