Anatomy of Associative Long-Term Synaptic Modification

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I. INTRODUCTION

Historically, the modification of connections within the nervous system is a favored theory to explain memory storage (Descartes, cited in Lashley, 1950; Spencer, 1862; Hebb, 1949) and for good reason. Comparison with other possible mechanisms, the modification of DNA for one, illustrates the many appealing aspects of memory storage by synaptic modification. First, there is the elegance of simplicity in changing an existing structure rather than synthesizing a new polypeptide specific for each new memory. Second, the existence of an extremely large number of synapses results in a nervous system with very high memory capacity if these synapses are "individually" modifiable. Certainly the memory capacity of the genepales in comparison. Finally, there is the placement of a synapse between two cells—a perfect location to "read in" and "read out" a summary of associated activity between the two cells. What better place to "sense" associative activity than at, or just next to, a synapse, and how simple it is to read out a memory—a synapse is just used again. By comparison, it seems problematic to relate other mechanisms, such as alteration of genetic material, to memory storage and retrieval. For example, even if one is able to have a large enough memory capacity, how would the specificity of the individual associative correlations be maintained without placing individual bits of genetic material at each synapse? If there is a biologically plausible answer to this question, we are unaware of it.

The idea of a synapse-specific form of associative modification receives strong support from recent experimental physiology (see Levy, 1985; Levy and Desmond, 1985b; chapters 2, 4, and 6 of this volume, for reviews). Such electrophysiological observations and the appeal of the synaptic modification hypothesis of memory storage (see below) motivated our anatomical explorations for synaptic modifications that would explain the changes observed electrophysiologically.

Anatomists have a long involvement with hypotheses of memory storage. With the advent of the neuron doctrine, some anatomists overcame the static appearance of fixed tissue to imagine morphotologically
based synaptic modifications of functional relevance. For example, Tanzi (1893) proposed that existing active synapses hypertrophy with increased synaptic activity. This hypertrophy would increase the influence of one cell upon another. Ramon y Cajal (1893) proposed a different, albeit related, concept, namely, that learning involved the formation of new synapses, with the growth of axonal and dendritic processes occurring to some extent throughout the life span of the organism.

These two hypotheses became increasingly provocative as more than half a century elapsed without experimental support for either idea. Now, however, there is strong experimental justification for both views—the modification of existing synapses and the formation of new synapses as some function of neuronal activity. Environmental enrichment (e.g., Diamond et al., 1975; see Greenough and Chang, 1985 for review), lesion-induced sprouting (e.g., Raisman, 1969; Lynch et al., 1972; see Cotman and Nieto-Sampedro, 1984; Steward, 1986 for reviews), electrical stimulation (e.g., Van Harreveld and Fiková, 1975; Rutledge, 1978), and behavioral conditioning (e.g., Horn et al., 1985) all produce morphologically identifiable synaptic modifications. Synaptic modification as a function of neural activity, therefore, is clearly an intrinsic property of the central nervous system.

Synaptic modifications that encode presynaptic and postsynaptic coactivity are also important to neurally based theories of cognitive function. The theoretical literature exploring the properties of distributed neural networks (e.g., Grossberg, 1976; Amari, 1977; Kohonen, 1977; Anderson, 1979) overwhelmingly emphasizes the role of associative synaptic modification in memory. Theories of learning and memory, including perceptual development (e.g., Bienenstock et al., 1982) and concept formation, would receive critical support if experiments could identify anatomical correlates of use-dependent synaptic modification. This support would be particularly valuable when the activity dependencies of the modification are precisely understood.

Our thinking has been greatly influenced by the various hypotheses and studies mentioned above. Additionally, there are at least three reasons, in our opinion, for taking an anatomical approach to study associative synaptic modification:

1. To identify subcellular loci of physiologically observed synaptic modifications
2. To delimit and enunciate sensible cell biological/biochemical hypotheses concerning the synaptic modifications
3. To provide anatomical correlates that can be applied to behavioral learning studies to localize the synaptic modifications underlying the behavior.

A variety of model systems are useful for understanding activity-dependent synaptic modification. Some of these are mentioned below.
However, this chapter focuses on the dentate gyrus of the hippocampus. Here the phenomenon of long-term potentiation (LTP) is associative (Levy and Steward, 1979) and can be reliably produced. The particularly simple anatomy of the dentate gyrus is important for interpreting the experimental results.

This chapter concentrates on some anatomical correlates of long-term potentiation (LTP) at the synapses formed by entorhinal cortical (EC) axons with the dendritic spines of the granule cells of the dentate gyrus (DG). The correlates of LTP discussed below include spine head size, spine head shape, spine stem parameters, number of spine synapses, and number of synaptic vesicles. Where appropriate, there is consideration of discrepancies between our results and anatomical correlates of LTP in other studies.

As suggested by the anatomical correlates discussed here, the basis of long-term synaptic potentiation at EC-DG synapses is a more potent postsynaptic event for the potentiated synapses because of changes specific to these synapses. The potentiation results from more transmitter release, more transmitter receptors, and altered longitudinal resistance of the dendritic spine stems at these potentiated synapses.

II. THE PHENOMENOLOGY OF LTP

A. Strong, but Brief, High-Frequency Stimulation Produces Potentiation

Bliss and colleagues (Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973) initially described the basic phenomenon of LTP at the EC-DG synapses. In the elementary LTP paradigm, brief trains of high-frequency conditioning stimulation applied unilaterally to the entorhinal cortical axons lead to a persisting increase in the amplitude of the synaptic response of the ipsilateral dentate granule cells to test stimulation of the previously conditioned afferent pathway.

LTP has since been observed in various other regions, including cerebral cortex (Komatsu et al., 1981; Lee, 1982; Wilson and Racine, 1983), cornu ammonis (CA) of the hippocampus (CA1: e.g., Schwartzkroin and Wester, 1975; Alger and Teyler, 1976; Andersen et al., 1980; Bliss et al., 1983; Barrionuevo and Brown, 1983; Lee, 1983; CA3: e.g., Yamamoto and Chuo, 1978; Misgeld et al., 1979; Yamamoto et al., 1980), other limbic forebrain structures (Racine et al., 1983), the medial geniculate nucleus (Gerren and Weinberger, 1983), superior cervical ganglion (Brown and McAfee, 1982), and the neuromuscular junction (Baxter et al., 1985).

In the EC-DG system and the Schaffer collateral-CA1 system, experiments reveal the associative nature of long-term potentiation (McNaughton et al., 1978; Levy and Steward, 1979; Wigstrom and Gustafsson, 1983; Barrionuevo and Brown, 1983). That is, production of synaptic potentiation requires a sufficient amount of presynaptic activity and postsynaptic excitation. For us, it is this associativity requirement more than the long-term nature of LTP that makes LTP important for system theories of emergent brain phenomena.
B. A Caveat: Synaptic Potentiation is Not the Only Change

There is a tendency to view LTP as a monolithic phenomenon. This tendency is not surprising since most studies use only one stimulating electrode with one test stimulation intensity and one recording electrode. In this situation, potentiation is the only observable effect.

Our view of long-term potentiation is somewhat different. Slightly more complex designs using multiple stimulating and recording electrodes, a variety of test intensities, and small variations in the conditioning stimulation procedure permit the observation of four distinguishable and dissociable long-term modifications in the dentate gyrus:

1. Long-term excitatory synaptic potentiation
2. Long-term excitatory synaptic depression
3. Long-term potentiation of the excitatory synaptic Input Transformed To Output (ITTO)
4. Long-term depression of the excitatory synaptic ITTO.

Electrophysiologically, modifications 1 and 2 are consistent with changes of the EC-DG synapses themselves (see Levy, 1985; Levy and Desmond, 1985b for reviews of the data supporting this interpretation). Excitatory synaptic potentiation in the dentate gyrus is an associative phenomenon requiring the correlated activity of excitatory afferents and sufficient postsynaptic excitation to increase the efficacy of the particular synapses involved (McNaughton et al., 1978; Levy and Steward, 1979; Wigström and Gustafsson, 1983). In contrast, when a particular presynaptic afferent is inactive concomitant with strong postsynaptic excitation produced by the activity of converging excitatory afferents, the efficacy of the synapse made by the inactive afferent upon the active postsynaptic cell decreases (Levy and Steward, 1979; Burger and Levy, 1983; Levy and Burger, submitted). In other words, there is long-term synaptic depression of the inactive, excitatory EC-DG synapse. Quantitatively, synaptic depression is as long-term in nature as synaptic potentiation (Burger and Levy, 1983). Both synaptic potentiation and depression have distinct temporal and spatial requirements (Levy and Steward, 1983; Levy et al., 1983; see Levy, 1985; chapter 4 of this volume, for reviews). Since associative synaptic potentiation does not decay merely as a function of time (Burger and Levy, 1983), synaptic depression may serve as a process to remove associative synaptic potentiation.

With care, modifications 1 and 2 are easily distinguished from 3 and 4. Most simply, measurement of the earliest phase of the synaptic response avoids effects related to cell firing. This is our typical experimental procedure.

Modifications 3 and 4, potentiation and depression of the ITTO, are observed electrophysiologically as potentiation and depression of the EC-DG population spike. Conditioning the EC projection to the dentate
gyrus increases the ITTO, even in the absence of excitatory synaptic potentiation (Wilson et al., 1979, 1980, 1981). Work by Abraham et al. (1985) is consistent with the hypothesis of Wilson et al. (1981) that the increase in the ITTO acts like decreased feed-forward inhibition. (Note that in this hypothesis interneurons would have a lower threshold, would fire first, and would not themselves show changes in their ITTO curves!) Concurrent conditioning stimulation of the EC-DG afferents and the inhibitory commissural projection to the dentate gyrus depresses the EC-DG ITTO (Brasel et al., 1982; Levy, 1984) and thus acts like potentiation of the feed-forward inhibitory synapses.

Our favored hypothesis to account for modifications 3 and 4 is changes in the efficacy of feed-forward inhibitory synapses in the dentate gyrus (see Levy, 1985; Levy and Desmond, 1985b, for reviews of the data supporting this interpretation). There are several anatomical changes that would plausibly be correlated with potentiation and depression of the ITTO. Perhaps most obvious is a change in the number and/or size of the inhibitory synapses on the dendritic cell dendrites. Another possibility is that the branching pattern of the granule cell dendritic tree changes with ITTO shifts. Ample evidence for such a change exists in other systems, including differential rearing (Uylings et al., 1978; Floeter and Greenough, 1979; Greenough et al., 1979; Juraska et al., 1980), sensorimotor cortical ablation (Standler and Bernstein, 1984), nerve transection (Rosenthal and C punches, 1984), and lesion-induced sprouting (Caceres and Steward, 1983). Note, however, that such anatomical changes may require a longer time interval than the observed interval between conditioning stimulation and the onset of potentiation or depression of the ITTO (a few minutes at most). Alternatively, there may be a rearrangement of excitatory synapses with potentiation and depression of the ITTO, an hypothesis suggested by Tsukahara's work in the red nucleus (Fujito, 1982; Tsukahara et al., 1982; Murakami et al., 1984). These hypotheses are experimentally testable in the EC-DG system, although as yet there are no data known to us.

There is no difficulty in physiologically distinguishing among these four phenomena and implying different underlying mechanisms [see Levy and Desmond (1985b) for a discussion of our electrophysiological dissociation of these changes]. In hypothesizing anatomical substrates, however, it is important to keep each hypothesis juxtaposed to the most sensible, physiologically measured modification. For example, our anatomical correlates focus on synaptic measures in a monosynaptic pathway because the corresponding electrophysiological observations focus on early monosynaptic excitatory synaptic potentiation and not potentiation of the ITTO. In the physiological experiments of synaptic modification, the measurements are made with a 1.8 to 2.3 msec delay from afferent stimulation; this is sufficient grounds for a monosynaptic interpretation. Thus, it seems most reasonable to seek anatomical changes at the level of the synapse that are related to the synaptic potentiation observed. On the other hand, the ITTO
effect, because it is an alteration of cell firing, can be delayed 5 to 10 msec from afferent stimulation. Thus, while reduced feed-forward inhibition can not explain synaptic potentiation, it could explain the ITTO shift. Hence, the anatomical correlates of the ITTO shift may reflect changes not in the excitatory synapses monosynaptically activated by the entorhinal stimulation but changes in the inhibitory synapses activated by these same EC afferents.

Using other mammalian brain regions for anatomical studies may not simplify the phenomenology of LTP. Because it takes some effort to see and to distinguish modifications 2, 3, and 4, using another system presently lacking documentation of 2, 3, and 4 may be only an illusory simplification.

In using an anatomical approach to studying activity-dependent synaptic modification, the multifaceted physiological observations of LTP suggest that there should be a variety of anatomical changes correlated with the variety of physiological changes. Often it is easy to separate correlates by using common sense. For example, enlarged excitatory synapses are sensibly the site of synaptic potentiation and not of synaptic depression. Similarly, smaller excitatory synapses are sensibly the site of synaptic depression.

Although the common sense approach is useful, ultimately another approach is necessary in order to correlate definitively anatomical changes with the known physiological changes. The alternative anatomical approach dissociates the variety of physiological modifications or differentially labels the sites of the different kinds of modifications. Such an approach requires a functionally relevant label that can be localized with precision to particular presynaptic elements, thereby allowing the distinction of active (potentiated), inactive (depressed), and unchanged EC-DG synapses. In other words, we need to be able to distinguish among potentiated, depressed, and unchanged synapses at the electron microscopic level. Until the advent of such a label, we will bias our experiments to produce as much synaptic potentiation as possible in order to concentrate our studies on this one phenomenon.

III. THE EC-DG SYSTEM AS A MODEL SYSTEM
A. Advantages of the EC-DG System for Anatomical Studies of Synaptic Modification

We are particularly interested in studying modifications that are synapse-specific and associative in nature because of the importance of these features to neurally based theories of cognitive function. Therefore, the primary requirement in our selection of a model system is the presence of associative potentiation and depression in that system. The long-term nature of associative potentiation and depression makes the phenomena easier to study. Associative potentiation/depression at the EC-DG synapses is well-documented, as is its long-term nature. Thus, the EC-DG system is a leading candidate as the system of choice. The extremely
simple and well-characterized nature of the EC-DG anatomy clinches the choice of which system to use as our model.

Perhaps the most conspicuous advantage of the EC-DG system is its simply organized and homogeneous anatomy. The principal neurons of the dentate gyrus, the granule cells, receive various extrinsic and intrinsic inputs that terminate in characteristic laminae in the dentate molecular layer. Conspicuously absent from the molecular layer are axo-axonic synaptic contacts; we have never observed such synapses in the tens of thousands of synapses studied to date. One extrinsic input, the entorhinal cortical input, is numerically if not functionally dominant. These afferents originate from a single cell type, the layer II stellate cells of the entorhinal cortex (Steward, 1976; Steward and Scoville, 1976). The EC axons form en passant synaptic contacts with the granule cell dendritic spines in the distal two thirds of the molecular layer (Hjorth-Simonsen and Jeune, 1972). In the anterior dentate gyrus, at least 93% of the spine synapses in the distal two thirds of the molecular layer originate in the ipsilateral entorhinal cortex, while 3–5% of the spine synapses there originate in the contralateral entorhinal cortex (Steward and Vinsant, 1983). Since the density of contralateral EC-DG synapses is greatest in the most anterior dentate gyrus, evaluation of slightly more posterior tissue sections provides a neuropil where the ratio of ipsilateral to contralateral EC-DG synapses is even more favorable for the observation of ipsilateral synapses. Thus, the vast majority of synapses in the distal two thirds of the dentate molecular layer are EC-DG spine synapses, providing a homogeneous population of synapses with which to study morphological changes accompanying LTP-inducing conditioning stimulation. In other words, when we evaluate changes in spine synapses in the distal two thirds of the molecular layer, there is the statistical assurance that the scored spine synapses are EC-DG synapses.

Another anatomical advantage of the EC-DG system is that quantitative descriptions exist of the granule cell dendritic trees and of the number and types of dendritic spines (see, e.g., Desmond and Levy, 1982, 1984a, 1985). This quantitation provides a useful baseline for studies of synaptic modification.

The EC-DG system is also well characterized electrophysiologically at both the extracellular and intracellular levels. Simultaneous intracellular and extracellular recordings in intact animals (Andersen et al., 1966; Lømo, 1971a, b) and in the in vitro tissue slice (see, e.g., Dudek et al., 1976; Fricke and Prince, 1984) allow a synaptic interpretation of extracellular recordings alone via potential divider theory (Klee and Rall, 1977). Furthermore, such recordings also demonstrate that the EC-DG synapses are of the classic excitatory type. That is, the EC-DG synapses excite the granule cells to fire action potentials by an increased conductance event that depolarizes the neurons.

A further advantage of the EC-DG system is the broad base of existing knowledge on the development of the dentate gyrus and anatomical,
physiological, and biochemical correlates of lesion-induced sprouting there (see Cowan et al., 1980; Steward, 1986, for reviews). Because these various studies of synaptic modification have all been done in the same structure, we can compare changes seen following brief, high-frequency conditioning stimulation with changes observed during development and sprouting. Such comparisons may allow the development of rules of synaptic plasticity applicable to all forms of synaptic modification (see Levy and Desmond, 1985a, for discussion of this point).

As with all systems, the EC-DG system has several disadvantages as a model system for studies of activity-dependent synaptic modification. A large body of evidence argues for glutamate or a glutamate-like substance as the transmitter at the EC-DG synapses (see Cotman and Nadler, 1981, for review). However, the multiplicity of receptor types (see Foster and Fagg, 1984, for review) and the reversibility of available ligands make biochemical interpretations complex and exact (i.e., submicron) receptor localization impossible at present. Furthermore, there is not as yet a great armamentarium of drugs as there is for the monoamine transmitters. The introduction of the N-methyl-D-aspartate (NMDA) receptor antagonist, 3-((+)-2-carboxy-piperazin-4-y1)-propyl-1-phosphonic acid (Davies et al., 1986) offers promise because of its greater potency and higher affinity for the NMDA receptor compared with existing compounds. However, future morphological studies of LTP will require specific, nearly irreversible ligands to allow receptor counts with subcellular localization.

A second disadvantage of the EC-DG system (and probably of many other model systems) is the multifaceted nature of LTP in the EC-DG system (see above). This disadvantage complicates experimental interpretations of the phenomenology and mechanisms of LTP unless care is taken. However, it is not impossible to dissociate these phenomena, and we have done so in many ways (Levy and Desmond, 1985b). Future anatomical studies must use these dissociations with marking techniques so that the mechanisms underlying each of the four electrophysiologically distinguishable changes can be clearly distinguished anatomically.

B. How Well Will the Results Generalize?

As biologists, it is always our hope that studies using a specific system will generalize to other systems. Since the EC-DG synapses are Gray type I synapses, there is the hope that the findings described here will generalize to other Gray type I synapses, e.g., those in the cerebral cortex.

Five structural and functional characteristics of the EC-DG synapses may determine how far their modification properties successfully generalize to other synapses:

1. An excitatory synapse, where synaptic activation produces an increase in conductance that leads to cell firing

2. Acidic amino acid neurotransmission (e.g., glutamate, aspartate, or related oligopeptides)
3. Asymmetric synaptic densities formed with dendritic spines as the postsynaptic elements (i.e., Gray type I)

4. Postsynaptic densities containing large amounts of the major PSD protein, a 50-kD Ca\(^{2+}\)/calmodulin-dependent protein kinase (see, e.g., Kelly and Cotman, 1978; Kelly and Montgomery, 1982; Kennedy et al., 1983)

5. The presence of NMDA receptors (Monaghan and Cotman, 1985).

(Note that some of these properties may be considered in tandem if their regulation is inseparably linked at the level of the gene; e.g., 4 and 5 seem like obvious candidates.)

By definition, the EC-DG synapses have all five of these characteristics. Other systems may possess these characteristics in differing numbers, and some systems may not possess them at all. In our view, it would be most extreme to generalize from the EC-DG synapses to the modification of inhibitory synapses. Not so extreme, but still questionable, is generalization to the excitatory synapses formed by granule cell parallel fibers with cerebellar Purkinje cell dendrites because of the relative paucity of major PSD protein in the postsynaptic densities there (Planagan et al., 1982; Miller and Kennedy, 1985) and also the relative absence of NMDA receptors (Monaghan and Cotman, 1985) as compared to the EC-DG synapses.

Another important difference among synapses may be the form of the permissive event for synaptic modification. This dependency may alter the precursor event to modification, but may not alter the biochemical and morphological form of the final modification mediating the altered synaptic strength. For example, excitation of perforant path-CA1 synapses can be permissive for the modification of Schaffer collateral synapses in CA1 (King and Levy, 1986; Moore and Levy, 1986). Such supervised modification could well be mediated by an active dendritic process that helps transmit a distant synaptic event down the dendrite. In contrast, synaptic potentiation at the EC-DG synapses can be characterized as the unsupervised modification of a homogeneous class of inputs that provide their own permissive event via simpler local summation processes (see chapter 4).

IV. OTHER MODEL SYSTEMS

The dentate gyrus is, of course, not the only model system that will be useful for understanding long-term potentiation. Here we mention just a few other model systems with a variety of advantages and disadvantages. For some systems, their simplicity may lead to more direct experimental measures and stronger conclusions than can be readily obtained in the dentate gyrus although their very simplicity may make it more difficult to generalize the results to cerebral cortical synapses. Other systems may eliminate the associativity aspect of potentiation in order to study presynaptic mechanisms involved in long-term synaptic modifications. Still other systems may be better for clearly distinguishing among
different classes of modifications that may often coexist, e.g., the modification of existing synapses and the formation of new synapses.

Several model systems are useful for studying processes involved in the formation of new synapses as opposed to the modification of existing synapses (see Levy and Desmond, 1985a, for discussion of this distinction as it relates to systems functions). These systems include the neuromuscular junction (see Hopkins and Brown, 1985, for review), where competition occurs during normal development and experimentally in mature animals, and the superior cervical ganglion (Wolff et al., 1979; Field and Raisman, 1985), where lowered spontaneous activity or inhibitory processes per se play a role in new synapse formation.

Previously, we (Levy and Desmond, 1985a) suggested that associative potentiation/depression involves processes quite similar to those involved in the developmental process called competition. If this were true, then polyinnervated neuromuscular systems may be ideal for studies of the biochemical and cellular processes that are the sequence of reactions and the substrate of associative potentiation/depression.

Associative modification is certain to be a critical part of classical conditioning. Aplysia offers a useful model system for neurochemical studies of classical conditioning (e.g., Hawkins et al., 1983; Carew et al., 1984; see Hawkins and Kandel, 1984, for review). Classical conditioning of the rabbit nictitating membrane blink offers a superb mammalian system for studying associative long-term modifications of cerebellar circuitry (see Thompson et al., 1984, for review). Based on differences in morphology, chemistry, and apparent activity dependencies of modification (Ito and Kano, 1982; Ekerot and Kano, 1985), the cerebellar model system may well generalize to a different class of synapses than do the EC-DG synapses. However, extrapolation from the results of Thompson's laboratory (Knowlton et al., 1985, 1986; Logan et al., 1985; Lavond et al., 1985; Steinmetz et al., 1985; Chapman and Thompson, 1986) suggests a basic similarity of activity dependencies with the EC-DG system.

Finally, there are a variety of simple neural systems showing long-term potentiation, although not all of them clearly demonstrate an associative requirement. LTP in the superior cervical ganglion and at the crayfish opener neuromuscular junction is primarily a presynaptic phenomenon resulting from increased transmitter release (Brown and McAfee, 1982; Briggs et al., 1985a, b; Baxter et al., 1985). Both of these systems provide simple model systems for evaluating presynaptic mechanisms involved in LTP.

In contrast, hippocampal region CA1 demonstrates associative long-term potentiation (Barrionuevo and Brown, 1983; chapter 2, this volume) and thus offers another model system in which to study processes involved in associative synaptic modification. LTP in the CA1 region can be studied in the hippocampal slice preparation, thus offering a decided advantage for intracellular analyses. However, it is not yet apparent that the simplicity of CA1 is sufficient for anatomical studies. For example, the most
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interesting change observed in CA1, an increased number of shaft synapses (Lee et al., 1980; Chang and Greenough, 1984), may not reflect the activated Schaffer collateral pathway since Schaffer-CA1 synapses are predominantly axospinous (Westrum and Blackstad, 1962). The slice preparation has the added difficulty of poor morphology in a large portion of the slice due to trauma incurred by slicing and incubation.

For studies of synaptic modification, the EC-DG system has four advantages when compared with the Schaffer collateral-CA1 system: 1) the EC-DG afferents and synapses have a much better lamination (personal observations of Golgi impregnated material), allowing activation of a relatively specific subset of synapses; 2) more EC-DG axons may be stimulated simultaneously because the EC axons bundle in the angular bundle; 3) there are ten-fold fewer interneurons in the dentate gyrus (Seress and Pokorny, 1961); and 4) the EC-DG synapses are quantitatively known to be far and away the statistically dominant synapses in the distal two thirds of the molecular layer (see above). Such quantitation is unknown to us for the Schaffer collateral-CA1 synapses. Thus it is possible that the greater synaptic heterogeneity of the CA1 neuropil could well account for the differing anatomical changes obtained in the Schaffer-CA1 and EC-DG systems.

A. Extending the EC-DG Model System

Although our aim is to understand the cellular bases of learning and memory, the synaptic modifications observed during development, lesion-induced sprouting, environmental manipulations, and direct activity-dependent modifications in the EC-DG system may all reflect the same family of modifications (see Levy and Desmond, 1985a for discussion). That is, with the exception of cell birth and cell death, the developmental processes of synapse formation may be considered identical to the adult synaptic modifications that subserve learning. In this view, lesion-induced sprouting in the adult organism is not a repair process, but an expression of the organism’s propensity for synaptic modification due to changes in neuronal activity.

V. ANATOMICAL CORRELATES

The intent of our anatomical research is two-fold: to gather anatomical evidence for the synaptic nature of the electrophysiologically observed modifications in the EC-DG system and to produce hypotheses for the cellular bases of the physiologically relevant forms of activity-dependent synaptic modification. Of particular interest are synaptic hypotheses of the cellular bases of associative potentiation/depression.

This section attempts to make as explicit as possible the thinking that goes into our anatomical research and to discuss the results obtained to date. Interwoven with some abstract ideas about this research are specific examples culled primarily from our own studies.

The anatomical changes discussed below are correlates of the evoked
synaptic potentiation of the EC-DG synapses. These correlates all support the hypothesis that changes in the strength of existing synapses accompany associative long-term excitatory synaptic modification but that synaptogenesis does not. While there is no proof that these anatomical correlates cause the observed physiological changes, the anatomical correlates provide both bounds and suggestions for sensible hypotheses concerning the cellular substrates of the physiological changes.

A. Thinking About Correlates

Correlative studies necessitate particular care in their interpretation, since not all correlates may be pertinent ones. This section reiterates the thinking involved in correlative studies as a backdrop for the anatomical correlates described in the next section.

There are three arbitrary categories of interpretations to which the observed anatomical correlates of LTP may belong:

1. The correlate is a long-term modification that is a "lasting" cause, or a fundamental substrate, of the synaptic modification.
2. The correlate is part of the sequence of reactions leading to the long-term modification(s) of 1 above.
3. The correlate is not a mechanism in the sense of 1 or 2 and might be called a "noncausal" correlate. For example, some noncausal correlates follow from a cascade of events so as to be rather distant correlates of LTP.

1. Examples of 1 above. Some anatomical correlates of LTP could directly reflect the long-term modifications that are substrate(s) of the electrophysiologically observed synaptic modification. These correlates might include more synapses (but do not) and morphological changes suggesting increased synaptic strength itself, e.g., more synaptic vesicles, more postsynaptic density surface area, and shorter dendritic spine stems. In fact, "bigger" synapses is the anatomical correlate of long-term synaptic potentiation discussed below. By bigger, we mean more synaptic machinery necessary for increased release, increased postsynaptic membrane conductance, and increased spine stem longitudinal conductance. Similarly, smaller synapses may be the anatomical correlate of long-term synaptic depression.

2. Examples of 2 above. Correlates of the reactions leading to the long-term modifications by definition include both biochemical reactions and cellular biological processes. Examples of such reactions might be depolarization, ion fluxes, enzymatic reactions such as triggered protein phosphorylation or protease activation, membrane fusion events, and cytoskeletal contractions that could in theory move membrane components as well as produce functionally relevant shape changes. Certainly all of these reactions are interesting and probably essential to the ability of a neuron to modify. However, in order to garner evidence that a particular correlate belongs in this category, it is necessary to have a
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technique that fixes the relevant process in the appropriate time interval. In particular, if LTP is induced seconds after conditioning stimulation, then these seconds define the required fixation interval.

3. Examples of 3 above. Noncausal correlates of the LTP-inducing conditioning stimulation vary in their degree of interest. Some of these changes could reflect type 2 correlates that are slow to reverse. Other changes could reflect increased intermediary metabolism, ion pumping, or any process restoring a depleted substance. These latter changes could be misinterpreted as a type 1 or 2 correlate. For example, vesicle depletion and mitochondrial alterations including increased Ca^{2+} sensibly follow from mere neural activity, but are not the easiest phenomena to envision as substrates of synapse-specific, associative long-term potentiation.

Particularly difficult to interpret are transitory changes that significantly outlast the period of LTP induction but lack the longevity of LTP. If there is a sequence of substrates mediating synaptic modification, then we would expect a sequence of type 1 correlates. Thus, the time of sacrifice after conditioning stimulation could influence the substrate(s) observed anatomically. There are, however, no data indicating that this is the case in the EC-DG system. Although such transitory changes are often considered as type 2 correlates, we think it safer to classify them as type 3 correlates until data and the need for such interpretation suggest otherwise.

Other noncausal correlates may reflect an interaction between LTP and the technical protocols whereby the procedure, e.g., fixation, might induce, rather than preserve, the correlate. It is possible that the concavity of the dendritic spine heads (see below) is one such noncausal correlate of synaptic potentiation. However, even this noncausal correlate is useful. Spine head concavity can now be tentatively used as a synaptic marker for LTP in less simple systems when our anesthesia and fixation methods are followed.

As a summarizing example of these varied correlates, consider the rather obvious but hypothetical sequences of reactions that might lead to LTP in the EC-DG system. A temporal association of synaptic glutamate and dendritic depolarization activates the NMDA receptor and its ionophore so that there is a large, synapse-specific influx of calcium. This influx activates Ca^{2+}/calmodulin-dependent protein kinase, and phosphorylation activates other proteins to lay down more postsynaptic membrane and to aggregate more transmitter receptors. A concomitant presynaptic expansion occurs through an interrupted and stabilized vesicle recycling process. Long-term potentiation then ensues via a stronger synapse (more release and more receptors), seen morphologically as an increase in the synaptic interface. Several aspects of this sequence of reactions might be detectable by electron microscopy if the events can be quickly fixed, e.g., calcium influx and various protein modifications that lead to enlargement of the potentiated synapse. These observations are type 2 correlates of LTP. If calcium is first sequestered by the cell and then fixed, we would
have a noncausal correlate (type 3), which provides the inference of a type 2 event (more Ca\textsuperscript{2+} near the FSD). Changes in protein activation or posttranslational modifications, e.g., activation of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase and intermediate protein phosphorylations, are often interpreted as type 2 correlates although their fixation time does not qualify them as such. Tissue fixed after the initial, transitory events would show a greater synaptic interface; this observation is an example of a type 1 correlate.

B. Observed Correlates in the EC-DG System

This section considers anatomical changes observed in the relevant lamina of the dentate molecular layer following delivery of LTP-inducing conditioning stimulation to the monosynaptic entorhinal cortical afferents to the dentate gyrus. There are six noteworthy anatomical findings:

1. An increased incidence of concave spine heads
2. Fewer synapses
3. An increased spine head surface area
4. An increased area of the postsynaptic density and interfacial region
5. Shorter and/or wider dendritic spine stems
6. An increase in the number of front-line synaptic vesicles.

Results 4 and 6 depend upon the observed postsynaptic concavity (result 1) as a marker for activity or modification. Result 5 depends upon observations from a restricted, or biased, sample of dendritic spines and needs replication using more representative sampling.

These observations come from the population of asymmetric spine synapses in the middle third of the dentate molecular layer. As discussed previously (Desmond and Levy, 1983), the middle third of the molecular layer, the termination zone of the medial EC axons, is the region of active EC-DG axons during test and conditioning stimulation (see Fig. 1). The fact that many changes occurring in the middle third of the molecular layer do not occur in the proximal and distal thirds of the molecular layer indicates that the observed changes are specific to the activated EC-DG synapses.

Five methodological features of our anatomical studies should be made explicit. First, the experimental techniques maximize the amount of synaptic potentiation. The evoked EC-DG responses in these anatomical experiments are larger than those used routinely in our neurophysiological experiments. Altering the configuration of the bipolar stimulating electrodes, with more bare metal and greater tip separation, accomplishes this change. Second, the amount of synaptic potentiation is measured in each experiment, with a 50% increase required for animals to be included in the anatomical analysis. Third, the anatomical analyses include only animals with no evidence of electrical seizures during the experiments. That is, if a hippocampus seized or even showed afterdischarges at any time, the
Fig. 1. An average current source density analysis illustrating the approximate location of the synapses in the dentate molecular layer activated by the angular bundle stimulation typically used in our anatomical experiments. Inward relative current indicates the location of the activated synapses. The activated EC-DG synapses are approximately localized to the middle third of the molecular layer. (Redrawn in part from Desmond and Levy, 1983)

experiment was discontinued. Fourth, in the second and third sets of animals, the control dentate gyrus received low-frequency test stimulation equal in number to the total number of stimuli in the conditioning trains the experimental dentate gyrus received. The anatomical changes observed in the conditioned dentate gyrus are then attributable not merely to electrical stimulation and the ensuing cellular activation, but to the brief, high-frequency conditioning stimulation and an apparently specialized form of cellular activity that leads to synaptic potentiation. Finally, double-blind scoring procedures are used throughout our studies, with the identity of the tissue being revealed only at the end of scoring.

1. Concave spine heads. The original, preliminary observation of an increased number of concave spine synapses with LTP was found in collaboration with Oswald Steward. Since our initial report (Desmond and Levy, 1983) and the analysis of other synaptic features, the strongest and most consistent anatomical correlate of potentiation is an increased number of concave dendritic spine heads (see examples in Fig. 2).

Three observations tie together the correlation between synaptic potentiation and the increased number of concave spine heads. First, the increased number of concave spine heads is restricted to the middle third of the molecular layer (Desmond and Levy, 1983). This is the region activated by the conditioning stimulation and is the presumed location of the potentiated synapses. Second, the increase is rapid. It takes place within 2 minutes following the last conditioning train and persists for as long as we have looked (60 minutes following the end of the conditioning stimulation interval; Desmond and Levy, 1986a). Third, the result is
Fig. 2. Selected photomicrographs from the conditioned and control sides of an animal surviving 10 minutes following brief, high-frequency conditioning stimulation that elicited an 80% increase in the slope of the population EPSP. Photomicrographs are from the middle third of the molecular layer, the activated region of the dentate neuropil. A) Conditioned side. B) Control side. Note the increased incidence and larger size of the concave (+) spine heads on the conditioned side as compared with the control side. Bar = 1 μm.
repeatable, beginning with the unexpected pilot observations and continuing in three independent groups of animals (see Desmond and Levy, 1983; 1986a). Thus the concave spine synapses are a correlate of potentiation of the EC-DG synapses in our hands.

An increased number of concave spine heads was not an observation in other studies of LTP in the hippocampal formation. The absence of such a result in the CA1 region of the hippocampus (Lee et al., 1980; Chang and Greenough, 1984) can be ascribed to differences in the two model systems, including more interneurons in CA1, the explant method, and some difficulty in localizing the active synaptic region in CA1 (see above).

The relationship between our results and those from Fišková's laboratory, also using the EC-DG system (Van Harreveld and Fišková, 1976; Fišková and Van Harreveld, 1977), is more subtle. The concavity of the granule cell dendritic spine heads appears to be a fixation-dependent observation. That is, using urethane-anesthetized animals fixed conventionally, we see concave spine synapses while their fast-freeze technique visualizes rounded spine heads. Arguments can be made either way as to which fixation method produces the more natural cytoskeletal fixation. The concern about cytoskeleton in the context of spine head shape grows out of thermodynamic considerations. A flexible lipid-based membrane like the plasma membrane tends to assume the shape of a sphere. In order to overcome this tendency, some additional force, such as a cytoskeleton linked to the plasma membrane, is necessary to deform the spine head into a nonspherical element. Tissue from the dentate gyrus prepared by fast-freeze methods does not contain concave spine profiles (see, e.g., figures in Fišková and Van Harreveld, 1977; Boyne, Levy, and Desmond, unpublished observations). Since cytoskeletal preservation appears minimal with a fast-freeze method that includes osmication, it is possible that spine heads round up due to osmication. That is, osmium causes cytoskeletal destruction, and the liquid state seen by the tissue during osmication provides an opportunity for membrane shape change. On the other hand, it could be argued that mixed aldehyde perfusion fixation does not just stabilize the existing cytoskeleton, but artificially polymerizes it beyond the amount normally present, thereby producing concave dendritic spine heads.

(An alternative suggestion for the postsynaptic concavity attributes the observation to a presynaptic change. That is, expansion of the presynaptic en passant bouton into the postsynaptic spine head alters the shape of the postsynaptic element. This notion is not appealing in an en passant system such as the EC-DG system because it leaves unexplained the localization of the presynaptic protrusion to the membrane apposing the dendritic spine head.)

We do not infer any specific functional significance to the postsynaptic concavity and view the result as a noncausal type 3 correlate of the conditioning stimulation procedure of LTP. (F. Crick and T. Brown (personal communication), however, postulate that such a change would
be useful for a retrograde, mechanical transduction across the synapse to alter, in some way, transmitter release.] Regardless, the observed concavity is a very useful marker for at least the activated, if not for the potentiated, synapses.

In this view, the postsynaptic concavity becomes a “label” for the conditioned and potentiated synapses, thereby permitting the selective evaluation of the potentiated population of synapses. Such a label allows a comparison between the conditioned and control sides of an animal in which the potentiated (concave) synapses in the activated region of the molecular layer are compared with all spine synapses in the corresponding region on the control side.

2. The interconversion hypothesis. How might the increased number of concave spine synapses in the region of synaptic activation with synaptic potentiation be explained? Since there is no increase in the total number of synapses in the region of synaptic activation (Desmond and Levy, 1983; 1986a), new synapses seem an unlikely explanation. Moreover, conditioning stimulation does not lead to an overall decrease in the number of synapses within other laminae of the molecular layer. Thus we cannot easily hypothesize that the increased number of concave synapses in the middle molecular layer comes from a migration of concave synapses from elsewhere in the molecular layer. There is, however, a concurrent decrease in the number of nonconcave spine synapses in the middle molecular layer with synaptic potentiation (Desmond and Levy, 1983; 1986a).

Our working hypothesis is that the increase in the number of concave spine heads results from a conversion of nonconcave spine heads into concave spine heads with LTP-inducing conditioning stimulation (see Fig. 3). In other words, associative activity leads to synaptic potentiation and to a conversion of spine synapses from nonconcave to concave. Certainly this is a conservative hypothesis compared to the alternative that these additional concave spine synapses appear de novo with conditioning stimulation, i.e., that synaptogenesis produces these concave spine synapses while resorption removes the lost nonconcave spine synapses.

The interconversion hypothesis requires the bold assumption that the increased concave spine population is, in fact, the population of spines that is potentiated or, at the very least, the population of spine synapses that receives high-frequency conditioning stimulation, a large fraction of which is also potentiated. We believe this assumption to be justified. Since our experimental design ensures the activation of large numbers of EC-DG synapses and large amounts of synaptic potentiation (see above), most of the activated synapses are also potentiated synapses.

The interconversion hypothesis is important for the interpretation of other anatomical correlates of LTP (see below).

3. Decreased number of synapses. As briefly mentioned above, there are actually fewer synapses in the middle molecular layer, the region of primary synaptic activation, with synaptic potentiation (Desmond and Levy, 1983; 1986a). Following conditioning stimulation, there is a 12%
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![Diagram of synaptic changes](image)

**Fig. 3.** Schematic drawing illustrating the proposed interconversion hypothesis. The hypothesis explains the increased number of concave spines in the activated region of the dentate molecular layer following LTP-inducing conditioning stimulation with no corresponding increase in the total number of spine synapses there. Before synaptic potentiation, a dendritic spine head is nonconcave. With brief, high-frequency activity, vesicle fusion occurs and the presynaptic membrane expands (see middle frame). At those synapses where the postsynaptic neuron is active concurrently with the presynaptic activity, associative potentiation occurs. Note the weakening of the spine stem cytoskeleton in the middle frame. The postsynaptic apposed membrane then expands at the potentiated synapses, stabilizing the presynaptic membrane expansion. The potentiated spine heads convert from nonconcave to concave in shape via condensation of additional cytoskeleton in these spine heads. Note that this hypothesis proposes an interconversion of existing synapses with LTP and does not require synapse formation to occur.

Decrease in the synapse density (number of synapses per unit volume of tissue) in the middle third of the molecular layer (control $N_v$ 2.56 synapses/$\mu m^3$; $t = 2.067$, $p < 0.05$). We believe that this statistically significant decrease in the number of synapses, although small, is real. Note that this is the same region of the molecular layer where the number of concave spine synapses increases, an increase hypothesized to reflect synaptic potentiation (Desmond and Levy, 1983; 1986a). Of course, the decreased total number of excitatory synapses is not the cause of LTP, although this anatomical change may reflect another physiological change accompanying LTP.

Since the decreased number of synapses reflects a change in the small, nonconcave population of spine synapses (Desmond and Levy, 1983, 1986a), the most consistent hypothesis is that the decreased number of synapses is a correlate of long-term synaptic depression. Recall that depression is the rule at those synapses with inactive presynaptic elements during the interval of net postsynaptic excitation (Levy and
Steward, 1979; see Levy and Desmond, 1985b, for review). Although it is not necessary that potentiation and depression be morphological opposites, it would be simple if one anatomical correlate of synaptic depression is smaller synapses. In theory, then, synapses could, if repeatedly depressed, become small enough so as not to be counted as well as becoming functionally useless.

4. Spine head surface area. The original correlate of LTP in the dentate gyrus is larger spine heads as inferred from two-dimensional areal measurements (Van Harreveld and Fišková, 1975; Fišková and Van Harreveld, 1977). We replicate this result (Desmond and Levy, 1983).

Perhaps not surprisingly, the bigger heads are the concave spine profiles. Even in control tissue, concave spines have a greater surface area than do nonconcave spines (see Fig. 4). With potentiation and the associated increase in the number of concave spine synapses, there is a corresponding increase in the number of large spine heads and a decrease in the number of small spine heads (Desmond and Levy, 1983). This result confirms the persistent increase in spine head size with potentiating stimulation of the EC-DG afferents previously observed by Fišková and Van Harreveld (1977).

The increased spine head surface area may be functionally important. Perhaps an inactive spine head is able to sink some of the current generated by other synaptically activated spine heads. The idea is that a potentiated, but synaptically inactive, spine (i.e., one with more surface area) would sink more current from presently active spines than it would have prior to its potentiation (i.e., when the spine had less surface area). This hypothesis (see Fig. 5 for schematic illustration) predicts that less current from active spines reaches the soma if neighboring spines are potentiated but presently inactive. This hypothesis may require a voltage-activated K⁺ conductance in the spine head to be functionally meaningful. Regardless of the exact requirements of the current sinking mechanism, this property: 1) is useful for some computational models of abstract neural function in which synapses store conditional probabilities that are multiplicatively combined (Levy, in preparation); and 2) may, in part, account for long-term synaptic depression.

5. Apposed membrane and postsynaptic density. Since total spine head surface area increases with synaptic potentiation, it is reasonable to expect that the membrane area lying in apposition to the presynaptic element also increases with synaptic potentiation, and this is the case. Quantification of this membrane area, termed the apposed membrane, shows a significant increase for the concave spine profiles in the middle third of the molecular layer with LTP-inducing conditioning stimulation (Desmond and Levy, 1984b; submitted).

Because the PSD delimits a distinctive portion of the apposed membrane that may have a high receptor concentration, changes in this delimited membrane area are of particular interest since these changes may imply changes in the number of transmitter receptors. The area
Fig. 4. Spine head surface area for concave (solid circles) and nonconcave (open circles) spine profiles in the middle third of the control molecular layer. Note that, on the average, concave spine heads have far greater surface area than do nonconcave spine heads even in control tissue. The circle size method (Desmond and Levy, 1983) used here is a two-dimensional measure of three-dimensional surface area. In fact, this result underestimates the size distinction between concave and nonconcave spine heads. Unpublished observations show that the circle size measurements are accurate within 92% for the nonconcave spine heads, but consistently underestimate surface area for the concave spine heads. The arrows indicate the 50% point in each distribution. Values plotted are the mean of six animals.

delimited by the postsynaptic densities of concave spine profiles increases markedly with LTP-inducing conditioning stimulation (Desmond and Levy, 1986b). Figure 6 is a histogram of observed PSD trace lengths for all spines in the middle third of the molecular layer. Note the shift in the distribution toward longer trace lengths with synaptic potentiation. This increase only occurs in the region of the molecular layer containing the activated EC axons. Since concave dendritic spines normally have larger PSDs and interfacial membrane regions than do nonconcave dendritic spines (Desmond and Levy, 1983; unpublished observations), the changes seen with potentiation appear to be an exaggeration of a normal, on-going process.

Based on the hypothesis that the PSD area or apassed membrane area delineates the number of synaptic receptors (see, e.g., Lester et al., 1981; Matus et al., 1981; Wu et al., 1985), the localized increases in interfacial membrane area and PSD area observed with synaptic potentiation suggest a correlated change in the number of postsynaptic neurotransmitter receptors. Unfortunately, the lack of nearly irreversible, specific ligands at present prevents us from performing the more conclusive biochemical receptor identification and localization experiments.
Fig. 5. Hypothetical changes in current flow in the dendritic spine as a function of potentiation. The left side of the schematic drawing represents two unpotentiated synapses, one synaptically active and the other not. The right side represents two potentiated synapses, one synaptically active and the other not. When an associatively potentiated synapse is active, the amount of synaptic current flowing into its parent dendrite increases (more and larger arrows). If this same potentiated synapse is now inactive while neighboring spine synapses are active, more current from the active spine synapses flows out of this large inactive spine (more and larger arrows). Thus, a potentiated synapse produces more current if active and sinks more current if inactive.

At this point, it is reasonable to ask from where the additional PSD and apposed membrane arise. The enlargement of the PSD surface area with synaptic potentiation may just result from condensation of slightly modified soluble proteins in the dendritic spine cytoplasm. For example, the major protein of the postsynaptic density is a 50,000-MW protein, identified as the major subunit of $\text{Ca}^{2+}$/calmodulin-dependent protein kinase (Banker et al., 1974; Blomberg et al., 1977; Kelly and Montgomery, 1982; Kennedy et al., 1983; Kelly et al., 1984). The ubiquity of $\text{Ca}^{2+}$/calmodulin-dependent protein kinase in the neuronal cytoplasm (1–2% of total brain protein; Erondu and Kennedy, 1985) lessens the need for rapid, on-demand synthesis of postsynaptic density proteins. Instead, it is feasible that this protein kinase is readily condensed directly from the cytoplasm into the PSD, perhaps via autophosphorylation (Shields et al., 1984; Miller and Kennedy, 1986).

Alternatively, preexisting PSDs may spread and thin with potentiation. This possibility has not yet been evaluated.

The origin of the new apposed membrane is another story. Because the total surface area of the spine heads also increases with potentiation, it is not satisfactory merely to propose that the increased interfascial membrane comes from spine head membrane existing prior to potentiation.

6. Whence does the membrane arise? How is it that the spine head is able to change its size, and what is the dynamic cellular process involved?
Fig. 6. Observed PSD length increases with brief, high-frequency conditioning stimulation. These normalized histograms illustrate the average distribution of PSD lengths for all spine profiles in the middle third of the molecular layer. Solid bars are values from the conditioned side; open bars are values from the control side. Following conditioning, some spine synapses shift into larger categories, i.e., more synapses have longer PSD trace lengths. Values plotted are mean ± SEM (N = 18 animals).

These are difficult questions to answer, particularly without freezing the dynamic process. It is quite likely that even our shortest experiments (in which the time between the final conditioning stimulation train and initiation of perfusion is about 60 seconds) are not fast enough to catch the bulk of such dynamic changes as an on-going process. Therefore, the following three hypotheses are highly speculative.

Perhaps some type of "Palade" vesicle fusion contributes membrane to the spine head (see arrow in Fig. 7). Unfortunately, finding such vesicles in spine heads is rare. Even when such vesicles are seen, the direction of membrane movement cannot be ascertained without a labeling method. That is, such an observation could just as well be a correlate of membrane removal as of membrane addition.

Another plausible mechanism is the direct transport of membrane from the endoplasmic reticulum to the spine apparatus and then from the spine apparatus to the spine head membrane. Serial section reconstruction reveals that the spine apparatus is attached to smooth endoplasmic reticulum as well as to the plasma membrane or the postsynaptic density (Spacek, 1985; our unpublished observations). How and where such membrane would be inserted into the plasma membrane are open questions. Since high-frequency electrical activity may be sufficient to modulate the topography of preexisting proteins in the postsynaptic membrane
Fig. 7. "Palade-type" coated vesicle fusion at the postsynaptic density of a connected spine synapse (arrow). Photomicrograph is from the middle third of a conditioned dentate molecular layer. The animal survived 60 minutes following conditioning stimulation. One mitochondrion in the presynaptic element apposed to this synapse appears to be fixed while recently active. (Compare its swelling to the state of other mitochondria in the field.) Bar = 1 μm.

(see, e.g., Fraser and Poo, 1982; Horwitz, 1984), perhaps such activity also influences membrane insertion.

Finally, and most appealing in its simplicity, new spine head membrane may come from the spine stem via cytoskeletal changes. That is, there is no new membrane inserted into the spine head, only a shape change that causes us now to identify as spine head membrane what was formerly spine stem membrane. The narrow cylinders of spine stem membrane are thermodynamically unstable, especially when compared to a sphere. Therefore, there is a requirement for something like a cytoskeleton to hold the cylindrical shape of the stem against the natural tendencies toward a spherical shape. By activating some process, e.g., a Ca^{2+}-activated protease that disconnects or destroys the cytoskeleton locally at the synapse side of the spine stem, the spine head enlarges and the spine stem shortens. [Note the weakening of the cytoskeleton (dotted lines) with associative activity and the shorter "after" spine stem suggested by Fig. 3. A Ca^{2+}-activated protease was suggested to play a role in LTP by Lynch and Baudry (1984) for other reasons.] This single process...
could account for both the increased spine head size and the decreased spine stem length correlated with LTP (see below).

7. **Dendritic spine stems.** Change in the dimensions of dendritic spine stems is an appealing mechanism for functionally relevant synaptic modification (see, e.g., Chang, 1952; Rall and Shepherd, 1968). Assuming that synaptic activation normally produces a low-resistance path through the spine head, the spine stem acts as a high-resistance, electrical bottleneck to current flow from an active synapse in the spine head to the parent dendrite. The hypothesis for synaptic modification is simple. "Synaptic" modification occurs via the modification of spine stem resistance. Lowering spine stem resistance by the stem growing shorter or wider produces potentiation; raising spine stem resistance by the stem growing longer or narrower produces depression. In other words, altering the spine stem dimensions adjusts the apparent synaptic current literally like adjusting the dimensions of a bottleneck changes the flow of liquid from a bottle.

Even though spine stem modification is not truly synaptic, in the Chang hypothesis there is no meaningful distinction between the modification of synaptic conductance and the modification of spine stem longitudinal resistance. A neurophysiologist with a somatic electrode or, for that matter, the soma and initial segment themselves effectively view any change in synaptically driven current due to altered spine stem dimensions as a synaptic change. Furthermore, since such spine stem changes provide a mechanism for individualized modification of apparent synaptic efficacy, many systems neurodynamical theories would consider an individual spine stem change as the modification of an individual synapse.

Rall's (1974) impedance-matching notion lends interest to and broadens the Chang hypothesis by explaining the apparently paradoxical existence of the longest and thinnest spine stems on distal cerebral cortical dendrites (Peters and Kaiserman-Abramoff, 1970) and distal granule cell dendrites (Desmond and Levy, 1985). The apparent paradox is that a distant dendritic locus weakens the influence of a synapse on the soma, and a long, thin stem makes a spine synapse even less influential for generating an initial segment spike. Thus distant synapses are apparently doubly weak. However, in Rall's hypothesis, it is desirable for the longitudinal spine stem resistance to match, within a ten-fold range, the resistance to ground measured at the junction of the stem with its parent dendrite (impedance matching). The physiological significance of spine stem changes and the inferred change in the ratio of spine stem resistance to branch input resistance depend on such impedance matching. The apparently paradoxical distal location of high-resistance spine stems allows for the optimal impedance matching of spine stem resistance and branch input resistance.

Potentiation based on spine stem shortening or widening requires that the spine stems in question normally have sufficiently high longitudinal resistance so that any decrease in resistance following potentiation suffi-
ciently alters current flow into the parent dendrite. Also required is an appropriate dendritic resistance to ground at the point of stem connection. Unfortunately, the normal ranges of spine stem longitudinal resistance and resistance to ground at various points in the parent dendrite are unmeasurable at this time. Approximations are possible by electrical circuit modeling using cell morphology and measurements of resistance to ground from the soma based on standard values of cytoplasmic and membrane resistivity. However, even these estimates may be misleading because of a naive view of the electrical characteristics of the spine. Dendritic spine membrane may well contain voltage-activated channels or other nonstandard dendritic membrane features (Shepherd et al., 1985; Miller et al., 1985; Perkel and Perkel, 1985). Moreover, the presence of a spine apparatus or numerous filaments within the spine stem may importantly affect its longitudinal resistance.

Such considerations make data correlating changes in dendritic spine stems in the dentate gyrus with potentiation suggestive, but problematic. Following conditioning stimulation, the diameter of "connected" dendritic spine stems increases in the dentate molecular layer (Fifková and Anderson, 1981). [Connected dendritic spines are those spines that are visibly attached to the parent dendrite in a single thin section. That is, there is a continuous image of dendrite, stem, and spine head with postsynaptic density. Of course, all spine synapses are connected. It is just that complete observation may require serial section reconstruction.] Work in progress in our laboratory supports the stem observations of Fifková and Anderson (1981).

The data on spine stem changes with potentiation are problematic for several reasons. Because connected spines represent such a small (ca. 10%) and selective fraction of the total population, these data can only weakly support any functional interpretation. Additionally, the changes observed may represent the shortening and widening of spine stems that are already quite short and wide. Such changes would have little, if any, influence upon the electrical properties of the spine stem because the spine stem resistance starts out relatively small compared with the branch input resistance, i.e., such spines already fall outside the ten-fold range for impedance matching (Rall, 1974). Further evaluation of the role of spine stem changes in synaptic potentiation requires thorough sampling of the entire distribution of spine stem lengths and diameters before and after conditioning stimulation. That is, how many stems changed, which spine stems changed, and how much did they change? This information, combined with estimates of the resistance to ground at appropriate dendritic locations within the molecular layer, allows more intelligent speculation about whether the magnitude of the spine stem changes is sufficient to account for the electrophysiologically measured potentiation.

Two reports, of LTP in the developing dentate gyrus and of LTP in the sprouted crossed EC-DG pathway when the dendritic spines are predominantly stubby in appearance (Duffy and Teyler, 1978; Reeves and Stew-
ard, 1987), weaken the case for the Chang hypothesis. Assuming responses in these studies are not dominated by a small subpopulation of synapses with long, thin stems, such stubby dendritic spines lie below the lower end of the ten-fold range required for impedance matching. Thus, changes in the spine stems in these two situations are not as likely to account for the observed LTP, provided the assumption is correct.

If the spine stem hypothesis fails as an explanatory device (i.e., as a mediator of long-term associative synaptic modification), then the existence of spine stems of variable lengths begs for another explanatory hypothesis. In building a new hypothesis, we need not stray far from the Chang-Rall idea that the function of a variably sized spine stem is variable spine stem longitudinal resistance. However, rather than the variation being related directly to resistance to ground and to a synapse's own quantitative ability to produce somatic depolarization, the variable spine stem may be better understood as a means of proportionately weighting each synapse's contribution to the polarization of a local dendritic region and as a means of controlling the interaction between spine heads. That is, one spine head can sink a neighboring spine's synaptic current. This quantitative ability would require proper matching of spine stem resistance. Thus, in this new hypothesis, control of spine stem resistance is critical in scaling the interactions among active and inactive spine heads (see Fig. 5).

8. Presynaptic changes. The release of exogenous glutamate, the presynaptic neurotransmitter at the EC-DG synapses, and of endogenous glutamate and aspartate increases following brief, high-frequency conditioning stimulation (Dolphin et al., 1982; Lynch et al., 1985; Eliss et al., 1986). An observation complementing this increased release might be an increase in the presynaptic vesicle population.

Changes in other presynaptic features may also correlate with synaptic potentiation. Proteins restricted to the presynaptic terminals, e.g., protein F1 or protein kinase C, change with LTP (Bar et al., 1980; Akers and Routenberg, 1985; Lovinger et al., 1985; Akers et al., 1986). Presynaptic dense projections, as quantified with ethanolic phosphotungstic acid (E-PTA), change with development of visual cortex (De Groot and Vrensen, 1978), visual training (Vrensen and Nunes Cardozo, 1981), sprouting (Hillman and Chen, 1985), and with activity (Triller and Korn, 1985). Perhaps the number of dense projections also changes with LTP-inducing conditioning stimulation. [The bismuth iodide method (Pfenninger, 1971a) is now the method of choice, however, since E-PTA does not visualize every synapse seen with conventional uranyl/lead staining (Burry and Lasher, 1978).]

The simplest presynaptic correlate would still be an increased vesicle density with LTP. Fifková and Van Harreveld (1977), however, found a decreased total vesicle density between 10 and 60 minutes following conditioning stimulation. It may be that the total number of synaptic vesicles per terminal is not the relevant anatomical observation to correlate sensibly with increased transmitter release.
Perhaps transmitter release draws from a subpopulation of the total vesicle pool. In particular, the population of synaptic vesicles closest to the presynaptic membrane and closest to the release sites may be the functionally relevant vesicle population. LTP of the Schaffer-CAl system correlates with an increase in the percentage of vesicles adjacent to the presynaptic active zone (Applegate et al., 1987). Data from our laboratory in the EC-DG system show that, although the total number of synaptic vesicles does not increase, potentiated spine synapses (the concave synapses) have more front-line synaptic vesicles than do control spine synapses (Desmond and Levy, 1986c).

If the front-line vesicles represent a readily releasable pool of transmitter, quantal content should increase as the number of front-line synaptic vesicles increases. Thus, the enhanced transmitter release reported by Bliss's laboratory and the enhanced synaptic efficacy measured electrophysiologically are in accord via this mechanism. However, if there are also more release sites per synapse, then an increase in the number of postsynaptic receptors may be required to maintain quantal size (cf. Baxter et al., 1985) because the increased release is distributed over a larger area. That is, quantal size that we presume is maintained would be determined not only by the amount of transmitter released but also by the ratio of receptors and uptake sites to released transmitter (as well as some consideration of passive diffusion processes and nonspecific binding).

VI. CONCLUSIONS AND A HYPOTHESIS

Our initial motivation to explore the anatomical correlates of long-term potentiation was, in part, to localize sensible, causal correlates of the electrophysiologically observed modifications. The findings consist of several synaptically localized morphological modifications, three of which can be simply related to synaptic function. These anatomical correlates indicate that excitatory synaptic potentiation may occur via three mechanisms:

1. More transmitter release
2. More postsynaptic transmitter receptors
3. More conductance through the spine stems of potentiated synapses.

A. Coordinated Modification

The three postulated mechanisms are not mutually exclusive; potentiation may well require a coordinated change in all three aspects of the synapse. Consider the following example. Before LTP, a single impulse activates a very large percentage of the receptors at an individual synapse. Furthermore, with such activation the spine head is driven very nearly to the reversal potential of the activated ionophores, as would be the case when longitudinal spine stem resistance is large compared to the transmembrane resistance of the activated spine head. In this example, we suppose that spine stem resistance lies within Rall's ten-fold range.
relative to dendritic input resistance at the interface of the spine stem and parent dendrite. Thus, spine stem resistance limits current flow through the spine stem.

In this example, neither increased transmitter release, more receptors, nor a combination of both changes produces LTP because receptor number is a limiting bound on the effects of release and the reversal potential is the limiting bound on the effects of receptor activation. If, however, the induction of LTP leads to a shortening and widening of the spine stem with no change in release or receptor number, synaptic potency increases. More interestingly, if all three changes occur at the same synapse with LTP, there is an even greater increase in potency because the increased spine stem conductance requires more active receptors to produce transmembrane conductance necessary to achieve reversal potential, and, further, more release is required to activate more receptors.

This coordinated change also allows the synapse to change again if associative events so demand. That is, a coordinated change in all three aspects of a synapse increases the dynamic range of morphological/biochemical alterations that produce physiologically significant synaptic modification. Thus, even if the spine stem resistance lies on the low side of Rall’s ten-fold range for impedance matching, further LTP can still occur via the other two mechanisms.

The coordinated change can be understood from another, more general viewpoint. Perhaps there is an optimal level of spine head depolarization with synaptic activation for a wide variety of processes at the synapse. In particular, we could hypothesize a set point where 1) a single presynaptic impulse results in transmitter release such that a fixed and large percentage of the postsynaptic receptors are active; and 2) the spine head is driven to the same potential before and after LTP. Thus, spine stem changes would require changes in release and receptors so as to maintain the set point.

B. Individual Modifiability

As we mentioned earlier, individual synaptic modifiability is essential for a high capacity, associative memory system that uses synaptic modifications for memory storage. Consequently, individual synaptic modifiability is of particular interest to us. The three anatomical changes emphasized here can easily explain how the expression and maintenance of modification is synapse specific. The explanation is simply that these anatomical changes are physically localized to individual synapses, and this location is where the modifications exert their primary effect. That is to say, it is an activated synapse with these anatomical changes that becomes stronger, not a neighboring synapse lacking these changes. The induction and maintenance of the anatomical changes are, in theory, easily compartmentalized by the structure of the spine synapse per se. The relative isolation of individual spine heads restricts the changes occurring
at a recently activated synapse to that synapse so that potentiation occurs at one synapse and not at its neighbors.

C. Speculation on the Dynamic Changes Leading to Modified Synaptic Potency

What events could induce synaptic modification in a synapse-specific manner? It seems that the mechanism producing the specificity of the modifications discussed above stems in large part from the mechanism producing the associativity requirement for LTP. Following the theme of others (Wigström and Gustafsson, 1985), the mechanism is the NMDA receptor complex. The dual requirements of transmitter release and postsynaptic depolarization (Dingledine, 1983) for strong activation of the NMDA-controlled Ca\(^{2+}\) channel, combined with the existence of many Ca\(^{2+}\)-triggered cell biological events, make the NMDA receptor complex a provocative candidate to explain associativity.

What particularly impresses us about the phenomenology of the NMDA receptor is the apparently delayed and prolonged activation of this receptor complex (Wigström et al., 1985). These characteristics are critical for a detailed explanation of this NMDA hypothesis. First, in order to account for both associatively produced LTP and the control experiments that do not produce LTP (Levy and Steward, 1979), there must be some way for the spine to distinguish among its own, solely receptor driven depolarization, depolarization driven by a depolarized dendrite when the synapse itself is not synaptically activated, and paired synaptic activation and dendritic depolarization. Synaptically generated spine depolarization would be quite brief and nearly to reversal potential given 1) low transmembrane resistance for the activated spine head relative to the longitudinal spine stem resistance and 2) high transmembrane resistance in the inactive state. Prolonged NMDA receptor activation would await and require a prolonged dendritic depolarization not provided by the transient, glutamate-activated spine head depolarization. By virtue of its prolonged nature, the dendritic depolarization is distinctive from the transient synaptic activation. In addition, prolonged NMDA receptor activation that can await the facilitating depolarization event would explain the timing characteristics of associative potentiation (Levy and Steward, 1983), in which the presumptive dendritic depolarization, when staggered by as much as 20–40 msec, must follow and cannot precede synaptic activation.

Note that the hypothesis here requires the NMDA receptor to be localized to the spine so that the Ca\(^{2+}\) influx event is also localized to the dendritic spine.

To repeat, the events associated by the synaptically located NMDA receptor complex are released glutamate (or a glutamate-like transmitter) and a prolonged, sufficiently large dendritic depolarization. These two events correspond to the requirements for presynaptic and postsynaptic activity, respectively, for associatively induced LTP. The requisite
dendritic depolarization may occur, physiologically, only with high-frequency presynaptic activity (Herron et al., 1986). The large postsynaptic Ca\(^{2+}\) influx (MacDermott et al., 1986) that results from the glutamate-activated, voltage-facilitated NMDA channel triggers other Ca\(^{2+}\)-dependent cellular processes within the dendritic spine. At least one Ca\(^{2+}\)-triggered event, the Ca\(^{2+}\)/calmodulin-dependent kinase of the PSD, is spatially adjacent to the synapse. [Interestingly, the positive feedback phosphorylation of Ca\(^{2+}\)/calmodulin-dependent protein kinase (Shields et al., 1984; Miller and Kennedy, 1986) offers an additional nonlinear process that may supplement the role of the NMDA receptor complex as arbitrator of associative modification (Stevens, 1986).] This and other Ca\(^{2+}\)-triggered, spine localized reactions would in turn produce the morphological and physiological alterations we and others observe with LTP. For example, Ca\(^{2+}\) influx could activate proteases (Siman et al., 1983) and alter the organization of actin filaments in the spine cytoplasm (see Fiffková, 1985, for review). These events would then lead to spine shape changes and to altered spine stem dimensions. In addition, such a sequence of enzymatic activations may lead to the anchoring of receptors in the postsynaptic membrane through such proteins as fodrin (Carlin et al., 1983) or calmodulin. In this regard, it is noteworthy that calcium and calmodulin both participate in the formation of receptor clusters in muscle cells (Feng, 1984). Additionally, there exists at brain synapses a 170-kD phosphoprotein with characteristics of a transmembrane glycoprotein (Groswald et al., 1983), which is phosphorylated by Ca\(^{2+}\)/calmodulin-dependent protein kinase (Kelly et al., 1985).

D. Retrograde, Transsynaptic Communication

Linking the transient increase of postsynaptic Ca\(^{2+}\) to a long-term increase of transmitter release presynaptically is, however, a little trickier.

The proposed solution is a stabilized expansion of the presynaptic and postsynaptic elements. The proposal entails the following three-step sequence leading to LTP. This sequence starts with multiple firings of a presynaptic axon temporally associated with sufficiently strong excitation of the converging, postsynaptic dendrite.

Step 1: Presynaptic expansion at the synaptic interface and just beyond
Step 2: Postsynaptic expansion of the spine head via partial dismantling of the spine stem
Step 3: Grappling irons and sticky stuff to stabilize steps 1 and 2 at the synaptic interface.

(We are metaphorical here in order to imply quite generally what is needed so that readers might first imagine their own specific cell biological hypotheses as opposed to the following speculations.)
In more cell biological terms, the sequence may be as follows:

Step 1: Presynaptic membrane spreading or expansion occurs as a natural consequence of transmitter release, i.e., via vesicle fusion. Paralleling and following from the observation that presynaptic activation without strong postsynaptic activation is not sufficient to produce LTP, presynaptic expansion would occur without steps 2 or 3. However, without the required postsynaptic depolarization and steps 2 and 3, the expansion is only transient because of normal vesicle recycling (Heuser and Reese, 1973). Thus, the hypothesis is that stabilization of the presynaptic expansion requires some quickly following event that is arbitrated postsynaptically.

Step 2: Postsynaptic expansion may be a consequence of altering the postsynaptic cytoskeleton, e.g., by a partial unraveling of the spine stem cytoskeleton and by addition of cytoskeleton and PSD to the distal spine head. This hypothesis assumes that total spine membrane surface area (spine head and stem) remains constant with LTP, i.e., stem membrane becomes head membrane due to cytoskeletal changes (see above). The additional cytoskeleton and PSD in the spine head provide some structural stability to the postsynaptic interface and allow it to match approximately the presynaptic expansion.

Presumably steps 2 and 3, as well as step 1, are Ca$^{2+}$-triggered.

Step 3: Steps 1 and 2 are stabilized into a "permanent" structural alteration by laying down an insoluble, intercellular bridge between the presynaptic and postsynaptic elements of the potentiated synapse. The substances for this bridge could come from the presynaptic cell, the postsynaptic cell, or somewhere else. Perhaps Shashoua’s ependymins, a group of glia glycoproteins (see Shashoua, 1985 for review), are one of these required substances. Relative to the present hypothesis, ependymins have two interesting features. First, ependymins are found in mammalian brain, with the greatest amounts in the hippocampus (Schmidt et al., 1986). Second, the deposition of ependymins is an event triggered by a sufficiently large Ca$^{2+}$ influx. Note, however, that this influx causes a localized extracellular calcium depletion that is the actual critical event for ependymin insolubility (Shashoua, 1984). Other macromolecules would be attached to ependymins. The long-term nature of the coordinated presynaptic and postsynaptic stabilized expansion then evolves from the insolubility of the junctional complex (Pfenninger, 1971b; Cotman and Kelly, 1980).

Of course, these cell biological hypotheses are in dire need of experimental observations.

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