Annu. Rev. Neurosci. 2001. 24:1071–89

MORPHOLOGICAL CHANGES IN DENDRITIC SPINES ASSOCIATED WITH LONG-TERM SYNAPTIC PLASTICITY

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Key Words LTP, hippocampus, learning, imaging, memory

■ **Abstract** Dendritic spines are morphological specializations that receive synaptic inputs and compartmentalize calcium. In spite of a long history of research, the specific function of spines is still not well understood. Here we review the current status of the relation between morphological changes in spines and synaptic plasticity. Since Cajal and Tanzi proposed that changes in the structure of the brain might occur as a consequence of experience, the search for the morphological correlates of learning has constituted one of the central questions in neuroscience. Although there are scores of studies that encompass this wide field in many species, in this review we focus on experimental work that has analyzed the morphological consequences of hippocampal long-term potentiation (LTP) in rodents. Over the past two decades many studies have demonstrated changes in the morphology of spines after LTP, such as enlargements of the spine head and shortenings of the spine neck. Biophysically, these changes translate into an increase in the synaptic current injected at the spine, as well as shortening of the time constant for calcium compartmentalization. In addition, recent online studies using time-lapse imaging have reported increased spinogenesis. The currently available data show a strong correlation between synaptic plasticity and morphological changes in spines, although at the same time, there is no evidence that these morphological changes are necessary or sufficient for the induction or maintenance of LTP. Still, they highlight once more how form and function go hand in hand in the central nervous system.

INTRODUCTION

The search for the mechanisms underlying learning in the brain spans more than a century. In the earliest form of this debate Cajal speculated that learning required novel neuronal growth (Ramón y Cajal 1893). At the same time, recapitulating an earlier suggestion from Spencer (1862), Tanzi (1893) argued that changes in

existing connections might underlie information storage in the brain. Later, Hebb incorporated both ideas into a postulate that suggested that alteration in synaptic strength, as well as formation of novel synapses, were responsible for memory storage (Hebb 1949, pp. 335).

In 1973 it was discovered that brief tetanic stimulation produced a long lasting form of synaptic plasticity, long-term potentiation (LTP), that can last for hours or days in the mammalian hippocampus (Bliss & Lømo 1973). Just before that the involvement of the hippocampal formation in memory was established by clinical data indicating that lesions of this structure in humans produce anterograde amnesia (Milner 1966). Since then, many laboratories have been studying LTP as a cellular model for information storage in the brain. Although the relation of LTP to learning is not universally accepted (e.g. see Mayford et al 1996 in favor and Zamanillo et al 1999 against it), LTP is a widely used and helpful paradigm for long-term synaptic plasticity in a central synapse. LTP furthermore nicely relates to neural network theories of brain function because it implements a local learning rule, an essential element for associative neuronal networks (Hopfield 1982), and one that ensures many of the computational features that make neural networks so attractive.

Although there have been reviews of the role of morphological changes of spines in LTP (e.g. Calverley & Jones 1990, Wallace et al 1991), a recent flurry of work, much of it using novel imaging techniques and time-lapse recordings, has added important information. In addition, there is now increasing biophysical evidence of the relation between morphological and functional parameters of the spine. We now know that the volume of the spine-head is directly proportional to the number of postsynaptic receptors (Nusser et al 1998) and to the presynaptic number of docked vesicles (Schikorski & Stevens 1999). Also, the small size of the spine head determines fast diffusional equilibration for calcium, whereas the length of the spine neck controls the time constant of calcium extrusion in spines (Majewska et al 2000a, Yuste et al 2000). This implies that the morphology of a spine directly reflects its function, and this makes it particularly relevant to investigate morphological changes of spines during synaptic plasticity.

MORPHOLOGICAL PLASTICITY OF DENDRITIC SPINES

Besides LTP, there are many different experimental or behavioral conditions that have been associated with changes in spine morphology that we will not be able to cover here in detail. Nevertheless, in the following we briefly mention some of these studies to help the reader approach this large body of literature. Many, but not all, of these studies indicate that increases of neural activity produce more spines. For example, light deprivation in mice causes a reversible reduction in the number of spines (Globus & Scheibel 1967; Valverde 1967, 1971). Similarly, increases in spine density occur after visual stimulation (Parnavelas et al 1973). Other environmental manipulations, such as rearing animals in complex environments, also alter spine morphology (Greenough & Volkmar 1973), so do social isolation (Connor &

Diamond 1982) and reportedly even space flight (Belichenko & Krasnov 1991). A reduction in the size of the spine has also been observed after the first orientation flight in honeybees (Brandon & Coss 1982). In birds, spine morphological plasticity is observed during postnatal development (Rausch & Scheich 1982), imprinting with light (Bradley & Horn 1979), and in learning tasks involving pecking (Patel et al 1988). Finally, in a fascinating study, squirrels have been documented to lose 40% of their spines during hibernation and to recover them in a few hours after arousal from hibernation (Popov & Bocharova 1992, Popov et al 1992).

Changes in spine form and number have also been observed in vitro. In dissociated cultures (Boyer et al 1998, Papa et al 1995) as well as in brain slices (Kirov et al 1999), pyramidal neurons have increased spine densities compared to those found in vivo. Pharmacological manipulations also influence spine morphology and number. Stimulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors is needed for the maintenance of spines, whereas blocking AMPA receptors reduces the number of spines (McKinney et al 1999). Alternatively, synaptic blockade with high Mg^{2+} and low Ca^{2+} increases both spine number and size (Kirov & Harris 1999). This is also found in cultured neurons disinhibited with bicuculline (Papa & Segal 1996) and after stimulation of internal calcium release (Korkotian & Segal 1999).

Early on it was noticed that spines are initially overproduced and later reduced in number during normal development and aging (Ramón y Cajal 1904). More recently it turned out that even during the estrous cycle of some mammals, large numbers of spines are produced in the hippocampus and later eliminated in substantial numbers (Woolley et al 1990). Finally, many diseases, such as dementia (Mehraein et al 1975), mental retardation (Purpura 1974), Down syndrome (Marín-Padilla 1972), irradiation (Brizzee et al 1980), malnutrition (Salas 1980), fragile X syndrome (Wisniewski et al 1991), and epilepsy (Multani et al 1994), can produce abnormalities in spine morphologies.

RAPID SPINE MOTILITY

Adding to the rich field of work that has documented spine morphological plasticity in different systems, it has been recently discovered that spines show considerable motility (Fischer et al 1998). This motility had been proposed by Blomberg et al (1977) and Crick (1982) and has now been documented in dissociated cultures (Fischer et al 1998), in brain slices (Dunaevsky et al 1999), and in vivo (Lendvai et al 2000). Rapid spine motility can produce changes in the morphological classification of spines (Parnass et al 2000), it is developmentally regulated (Dunaevsky et al 1999), and it is more pronounced during the critical period (Dunaevsky et al 1999, Lendvai et al 2000). The mechanism responsible for this rapid motility is actin-dependent (Fischer et al 1998, Dunaevsky et al 1999), is interfered with by volatile anesthetics (Kaech et al 1999), and could involve the Rho family of small GTPases (Tashiro et al 2000). Clearly, spine motility occurs throughout the central

nervous system (Dunaevsky et al 1999), but at present its function is still a mystery (but see Majewska et al 2000b).

EFFECTS OF LONG-TERM POTENTIATION ON SPINE MORPHOLOGY

As indicated above, in this review we focus only on LTP or synaptic stimulation experiments from mammalian hippocampus. We do not intend to provide a comprehensive overview but instead concentrate only on a few studies.

Long-Term Potentiation in the Dentate Gyrus

Fivkova Studies: Swelling of Granule Cell Spines After Tetanization Although long-lasting potentiation of the myotatic reflex in the partially deaffarented mammalian spinal cord was reported by Eccles & McIntyre (1953) it was Lømo and Bliss's description of LTP in the monosynaptic connections between the perforant pathway and the granule cells in the hippocampus that triggered enormous interest, up to present times, in the investigation of the morphological consequences of long-term synaptic enhancement in the central nervous system (Lømo 1970, Bliss & Lømo 1973).

Some of the earliest studies of the effect of LTP on the morphology of dendritic spines were carried out by Van Harrefeld & Fifkova (1975). They used an experimental protocol similar to that pioneered by Bliss and Lømo. The authors set out to test the hypothesis that LTP involved swelling of dendritic spines and reduction in neck length to produce increased synaptic activation by the input. By using a rapid freeze-substitution electron microscopy protocol that largely preserves spine morphology, the authors compared granule cell spines in the distal region of the dentate gyrus (the region that receives perforant pathway inputs) with those in the proximal region, devoid of such inputs, in both stimulated and control mice. By comparing distal and proximal spines from the same dentate gyrus, the authors controlled for potential artifacts and variability introduced by the experimental techniques. Taking samples from a narrow (10–15 μm) layer of freeze-substitute material, the authors used paper cutouts to measure the mean projected area of 100 spines. They found that in control animals distal spines had on average 13% larger areas than proximal spines, but that difference increased to 53% in stimulated animals. This effect was present as early as 2 minutes and lasted at least 60 minutes after stimulation, indicating that, similar to the synaptic enhancement produced by LTP, the increase in volume was immediately produced by the tetanization and was long lasting. The authors speculated that the increase in volume was produced by swelling due to the uptake of electrolytes, a mechanism previously proposed to underlie memory formation (Diamond et al 1970, Rall 1970). To further explore the relation between synaptic stimulation and spine swelling, in a second study the same authors carried out a temporal characterization of the effect and extended

their observation time to up to 23 hours (Fifkova & Van Harrefeld 1977). Again, they observed an increase in the volume of the spines from stimulated animals compared with controls. This increase was significant at all times tested (2 minutes to 23 hours), although it was largest (39%) 10–60 minutes after the stimulation. In the same experiments no systematic differences were observed in the proximal spines between stimulated and control animals.

The effect of tetanic stimulation on the size of the spine neck was specifically examined by Fivkova & Anderson (1981). Again, this study found an increase in the volume of the spine head in distal spines after tetanic stimulation. In addition, they found major increases (up to 42%) in the width of the spine neck, as well as shortenings (as much as 31%) of its length. These changes were again specific to distal spines and were present as late as 90 minutes after the stimulation.

Taken together, these studies paint a consistent picture of the effects of tetanizing the perforant pathway: The spine heads in the distal region of the dentate gyrus become larger, while the spine necks become shorter and wider. Although these studies did not confirm electrophysiologically the LTP presumably produced by the stimulation protocols and whether the exact spines analyzed were the ones that had been tetanized, the anatomical changes were long lasting and restricted to distal spines receiving perforant path inputs, just as one would predict from the properties of LTP. Fivkova later proposed that the enlargement of the spine head was in fact a result of an increase in the size of the synapse and that its ultimate stabilization might be mediated by the mobilization of actin caused by local increases in $[Ca^{2+}]_i$ (Fifkova 1985).

Desmond & Levy Studies: Further Evidence for Spine Modifications After Long-Term Potentiation Conclusions similar to the Fifkova studies were drawn by Desmond and Levy from a series of combined electrophysiological/morphological experiments (Desmond & Levy 1983; 1986a,b; 1988). They used the rat perforant pathway/dentate gyrus as the model system and combined field potential recordings and quantitative electron microscopy (EM) to search for changes in size and number of synapses after LTP. In these blind studies, two stimulation protocols were used: either a short burst of stimuli (massed conditioning) or a more distributed protocol with the same number of electrical shocks but spread out over time (spaced conditioning). The criterion for the inclusions of the animals in the study was a successful change of at least 50% in the initial excitatory postsynaptic potential (EPSP) slope, a relatively strict cutoff confirming the effectiveness of the physiological manipulation.

Desmond & Levy found anatomical changes following the two forms of conditioning, with up to 48% increases in the density in concave spines and up to 23% decreases in the number of nonconcave (simple and ellipsoid) spine profiles (1983, 1986a). Concave spine profiles, in their nomenclature, correspond to the population of large spines that have spinules or U-shaped profiles. These changes were more pronounced in the middle third of the molecular layers, the dentate gyrus region that was stimulated by their more medial electrode placement in the

perforant pathway. Although Desmond & Levy initially observed increases in the shaft synapses (1983), in their later studies they failed to detect significant changes in the total density of shaft or spine synapses (1986a).

These observations were further extended in a quantitative study of the size of the postsynaptic densities (PSD), in which the total PSD surface area per unit volume in concave spines increased significantly, whereas the PSD area of the nonconcave spines decreased (Desmond & Levy 1986b). In addition, the mean PSD length increased significantly across all spine profiles in stimulated animals and persisted for at least 60 minutes. In a later study the authors found concomitant changes in the presynaptic terminals (Desmond & Levy 1988).

These data showed increases in spine size after LTP, but at the same time other groups provided evidence for increases in spine number (see below). This prompted Desmond & Levy to consider the possibility of an increase in the number of synapses in the dentate gyrus (1990). The authors used two morphological markers of synaptogenesis, the presence of polyribosomes and multiple synaptic contacts, and found statistically significant decreases in their incidence after LTP, arguing for a modification of existing synapses, rather than de novo synaptogenesis. This result was extended to somatic ribosomes in a later publication from the same group, indicating that LTP is associated with increased protein synthesis (Wenzel et al 1993).

In conclusion, the Desmond & Levy studies—like the ones from Fifkova—present a scenario where there is an interconversion of spine shapes during LTP, without addition of new synapses. Smaller spines become larger spines, and this is mirrored by increases in the size of the synaptic surface area. In agreement with Tanzi's early suggestion, these data argue for no new synapses but for modification of existing ones.

CA1 LTP

Effects of Long-Term Potentiation on Synapse Localization Fifkova et al did not establish whether the stimulation protocols used indeed produced long-lasting synaptic plasticity. Therefore, Lynch's laboratory early on undertook several studies combining electrophysiological recordings with EM, examining the morphological effects of synaptic plasticity (Lee et al 1979a,b; Lee et al 1980). These experiments were carried out stimulating the Schaffer collateral pathway in vivo (Lee et al 1979b, 1980) or in vitro (Lee et al 1979a) and used blind analysis of ultrathin electron microscopic sections of the CA1 region of the hippocampus to compare the effects of low-frequency stimulation, which did not produce any long-lasting potentiation, with high-frequency stimulation, which produced LTP. After in vivo as well as in vitro stimulation, the authors found 33–50% more synaptic contacts on dendritic shafts in high-frequency stimulated animals. No significant differences were found in the number of synapses on spines, length of the spine PSD, area of spines, width of spine necks, or length of the PSD on the dendritic

shafts. Nevertheless, in the potentiated group the authors found a reduction in the variance of several morphological features of the spines. These studies did not confirm the swelling of spines or shortening of spine necks described by Fifkova and coworkers (Van Harrefeld & Fifkova 1975, Fifkova & Van Harrefeld 1977; Fivkova & Anderson 1981).

The authors speculated that LTP might be associated with increased innervation of dendritic shafts. This increase in shaft synapses could have been produced by retraction of spines or by new formation of shaft synapses, although they recognized that most shaft synapses were located on dendrites from relatively aspiny cells, i.e. inhibitory interneurons. Therefore, although these studies have the strength of electrophysiological monitoring of the effects of the stimulation, the conclusion drawn from their EM data are difficult to interpret.

A similar study was carried out by Chang & Greenough (1984), who also used hippocampal brain slices and ultrastructural analysis to compare the effects of different protocols of stimulation, including high-frequency stimulation that produced LTP, high-frequency stimulation that did not produce LTP, and low-frequency stimulation and synaptic inactivation by high-Mg^{2+} and low Ca^{2+} ACSF. After LTP the authors found significant increases in the number of sessile (stubby) and shaft synapses, whereas no detectable differences were found in the total number of spines or perforated synapses. Also, statistical reductions were observed in average spine perimeter, contact length, and the percentage of "cup"-shaped synapses, although no significant changes were encountered in spine head area, bouton area, PSD length, spine neck width, or length and number of presynaptic vesicles. These authors again concluded that LTP produced an increase in the synaptic innervation of interneurons, although they raised the possibility that it also induced the transition from shaft to spine synapses in CA1 pyramidal cells. These two effects would explain the increased number of synapses on dendritic shafts and stubby spines. They also proposed that LTP produced a change in spine shape, in which typical cup spines are transformed into a flatter spine profile with reduced spine perimeter and contact length.

INCREASED SPINOGENESIS AFTER LONG-TERM POTENTIATION: Andersen et al Studies

In all the studies discussed so far the authors reported rather subtle changes in spine shape or the geometry of the postsynaptic density. Andersen and coworkers were the first to report unexpectedly large increases in the number of spines as well as rather dramatic changes in spine shape. The initial studies (Andersen et al 1987a,b; Trommald et al 1990) were all done by inducing LTP in the dentate gyrus. Serial reconstructions of electron-microscopic material showed an increase in spine number of up to 50% as well as changes in the diameter of the spine neck and an increase in so-called bifurcated spines. These are structures hypothesized

to result from a splitting of a single synapse, first into a larger and supposedly more effective "perforated synapse" (Peters & Kaiserman-Abramof 1969) and from there into two synapses on different spine heads residing on a single spine trunk. Because it had been proposed that perforated synapses would be particularly effective synapses, the observation of increased numbers of such structures and bifurcated spines naturally explained an increased synaptic efficacy.

A later study by the same authors (Trommald et al 1996) elaborated on these initial findings and found that the strong effect of an increased spine density and more bifurcated spines indeed held up. The overall density of spines increased by ~30% while the number of bifurcated spines rose by a factor of more than three. Whereas this part of the earlier studies was corroborated, the changes in spine dimensions could not be confirmed. The authors reported that no statistically significant changes in the dimensions of the spines could be observed (Trommald et al 1996). This is not entirely unexpected, considering the enormous variation of shape and size in the dendritic spines that the authors report. Moreover, this result does not mean that such changes do not occur; it merely indicates that this (statistical) approach does not yield a significant result. The authors indicated that a method allowing one to observe single spines in living tissue over time would be much better suited to bring out potential morphological changes of this sort. They also noted that their results on the large increase in spine number is at odds with earlier studies that reported no increase in spines density (Desmond & Levy 1983) or even a decrease (Geinisman et al 1991). However, they have no obvious explanation for these differences other than variations in the experimental and stereological detail of the studies.

The same authors later used confocal microscopy instead of electron microscopy to further investigate morphological correlates of synaptic plasticity (Moser et al 1994). The reason for using confocal microscopy was presumably that it is much less tedious than serial EM. This in turn allows one to analyze many more spines and therefore allows for better statistical sampling to bring out small changes. In these new studies plastic changes were not induced by electrical stimulation but by an altered sensory environment, and the morphological changes were assessed in the CA1 region of the hippocampus. Rats were housed either alone or in pairs in plastic cages, and the experimental group was allowed to explore an environment with multiple platforms containing interesting items such as wooden blocks, branches, paper bags, and leaves. The animals that had the exploratory experience performed better in behavioral tasks like the Morris watermaze, but they also showed a small but significant increase in spine density, whereas all other measured parameters were unaffected. Interestingly, the enhanced spine density only occurred on basal dendrites (Moser et al 1997) and not on apical dendrites, which were also evaluated.

These studies are different from all the others discussed in this review in that the procedure applied is not necessarily expected to cause "storage" of new information; it is rather a skill that is acquired. Because the induction of LTP is often

thought to electrically mimic information storage in the brain, the aforementioned studies only indirectly relate to morphological changes observed with LTP. They suggest, rather, that acquiring the capability to learn better (and not the storage of information per se) can result in morphological changes in the form of newly emerging spines.

UNALTERED SPINE SHAPES AND NUMBERS: Harris et al Studies

A number of years later Harris and co-workers (Sorra & Harris 1998) tested whether tetanus-induced LTP would also result in an enhanced number of synapses in the CA1 region. Again they used an unbiased volume sampling procedure to look for possible changes in spine density or in the geometrical parameters of preexisting spines. In line with other investigators (e.g. Lee et al 1980, Chang & Greenough 1984), they found no changes in absolute spine number. This contrasts with the findings by Andersen et al (Andersen et al 1987a,b; Trommald et al 1990), who observed that at least in the dentate gyrus in vivo such changes seem to occur. Two major differences between these studies could contribute to this difference: (*a*) The brain area investigated was different in the two studies (dentate gyrus versus CA1 region) and (*b*) the Sorra & Harris study was performed in slices, whereas the others were performed in vivo. In this respect a follow-up study (Kirov et al 1999) is interesting and worrisome at the same time: These authors found that hippocampal slices showed, one hour after the slicing procedure, roughly two times more dendritic spines than "native" perfusion-fixed hippocampus. A similar result had been reported in somatic spines from granule cells from the dentate gyrus after slicing (Wenzel et al 1994). Therefore, a possible explanation for the lack of changes in the CA1 region of slices is that so much spinogenesis had occurred after the initial preparation that additional changes were either not possible anymore or "buried" within the noise.

Interestingly, Andersen's group (Andersen & Soleng 1998) verified that the enriched environment causes spinogenesis, and they also replicated Sorra & Harris (1998) findings showing that after 4 hours of LTP no net increase in spine number is observed in CA1 of hippocampal slices.

From all these studies it is clear (and in many of them it was explicitly noted) that online observation of potential morphological modifications would be a major technical improvement in the ability to detect potential changes. It would obviate the requirement to do statistics on large samples of experimental and control tissue and at the same time use temporal correlation of observed morphological changes with the experimental manipulations as a sensitive measure to detect causality. In fact, it is clear that certain kinds of morphological changes are only detectable by online observation: For example, statistical methods have no way of excluding that an overall null change of spine density is actually caused by some new spines

forming as a consequence of LTP and that this net gain of spines in the potentiated region is compensated for by the loss of spines elsewhere.

THE FIRST LONGITUDINAL EXPERIMENTS: The Hosokawa, Rusakov, Bliss, and Fine Study

Another important drawback of most studies reported so far is that they all relied on comparison of different populations of cells from different animals in fixed tissue, thereby creating a number of potential artifacts. Therefore, time-lapse imaging of living tissue was clearly an important step forward in investigating morphological changes after LTP.

Hosokawa et al (1995) were the first to move into this experimentally challenging direction. They used confocal microscopy of hippocampal slices in which individual CA1 pyramidal cells were stained by a specially developed "DiI-microdrop technique." Synaptic potentiation was induced by "chemical LTP," produced by the application of a superfusion solution containing elevated Ca^{2+}, reduced Mg^{2+}, and tetraethylammonium. The reason for choosing this kind of LTP induction was to cause potentiation in as many synapses as possible. Using this experimental approach, the authors observed that a subpopulation of (small) spines extended, and they further reported that there was an increased range of angular displacement of spines in the potentiated tissue. All other parameters showed no significant changes. In particular, the appearance of completely new spines was a rare (and statistically insignificant) event.

In principle, the resolution of (one-photon) confocal microscopy should be able to resolve these events. However, this technique is fraught with the problem of bleaching and photodynamic damage so that in practice, especially in longitudinal studies in living tissue, the attainable resolution is limited and just barely sufficient to detect and quantify spines under physiologically acceptable conditions. Another problem is that confocal microscopy can, due to the limited penetration of visible light, only detect superficial structures. Therefore, all synapses observed had to be within 75 μm (sometimes as close as 25 μm) of the surface, a depth of the slice at which there might be damage from the preparation of the tissue.

IMAGING SPINOGENESIS "ONLINE": The 1999 Studies

The problems of photodynamic damage and depth of penetration were largely overcome by the development of two-photon microscopy (Denk et al 1990). This method provides an ideal tool to further investigate morphological changes of spines that might be associated with LTP. In 1999 two independent two-photon studies on this question were published. In the first, Maletic-Savatic et al (1999) used organotypic slices and local stimulation to address the question of spinogenesis in this preparation. Individual cells were visualized by infection of the slices with sindbisvirus-eGFP constructs. These authors observed that a strong

tetanus, a stimulus that would normally induce LTP, led to local outgrowth of dendritic processes. The newly formed protrusions, however, were more akin to filopodia in that they were often >4 μm long. In some instances, however, these filopodia later turned into spine-like structures. Importantly, the emergence of new protrusions could be blocked by agents, such as APV, that interfere with LTP.

Shortly afterwards another study showed that new spines can be formed in hippocampal tissue even with much more moderate stimulation than the rather strong tetanus. This study (Engert & Bonhoeffer 1999) used a different strategy to pinpoint the locations where potential morphological changes could occur: In a relatively thin, different type of organotypic slice culture (Gähwiler 1981), all synaptic transmission was blocked by applying a medium with a relatively high concentration of Cd^{2+} ions and low Ca^{2+}. This transmission blockade was then relieved only very locally (in an area of $\sim$30 μm diameter) with a local superfusion system applying regular recording medium. This enabled the experimenters to know where the observed changes in synaptic strength must have taken place and therefore allowed them to scrutinize this area for morphological changes with a two-photon microscope while simultaneously monitoring the effect of the synaptic stimulation electrophysiologically. In every instance of successful synaptic enhancement, new spines were generated, whereas practically no new spines appeared when the enhancement was not successful (or blocked). This study therefore proved a strong correlation between the functional enhancement of synapses and the generation of new spines. These observations do not, of course, preclude that other more subtle changes in the shape of spines might also have occurred, but they clearly show that spinogenesis is a correlate of successful enhancement.

Later the same year an EM study was published that once more addressed the question of spinogenesis after LTP, but from a different angle. This study (Toni et al 1999) was an extension of earlier work from the same laboratory (Buchs & Muller 1996) using a technique to select for the stimulated synapses and therefore the location in which morphological changes were expected to occur. The assumption they used is that synapses that have just been subjected to a strong stimulus show, in electron-microscopic images, an accumulation of a calcium precipitate in the postsynaptic spine. This precipitate is visible postsynaptically and therefore "earmarks" spines activated by the electrical stimulation. Toni et al (1999) then scrutinized these spines for morphological changes and found that in many cases after stimulation there were pairs or triplets of (calcium precipitate–marked) spines making contact with the same presynaptic terminal. This then was taken as evidence that under conditions of LTP-inducing stimulation new spines were generated.

Although these studies have now established that there is a clear-cut correlation between the formation of new spines and successful enhancement of synapses, these results have to be interpreted with caution in that they show a correlation, but they do not prove causality in the sense that they do not prove that the newly observed spines with their presumptive synapses actually contribute to the strength of the measured connection . The study by Toni et al (1999) shows that in most cases

the new (second) spine contacts the stimulated axons and not other unstimulated ones. Therefore, the new spines could at least potentially contribute to enhanced transmission of the stimulated pathways. Yet, the experiments of Maletic-Savatic et al (1999) and Engert & Bonhoeffer (1999) actually prove that the emergence of new spines cannot be causal, at least for the early phase of the enhancement because the enhancement occurs within minutes, whereas new spines only appear after roughly 30 minutes. However, recent studies have made the interesting observation that interfering with the actin cytoskeleton, which among many other effects could presumably also inhibit the generation of new spines, can block LTP and even its early components (Kim & Lisman 1999, Krucker et al 2000).

Therefore, after two decades of searching for morphological correlates of LTP, it seems clear that such correlates exist. The role of these morphological changes, however, is still enigmatic. Direct contributions to synaptic strength as well as other functional roles, such as the generation of new potential contact sites or better calcium compartmentalization, are just a few of many possibilities.

CONCLUSION: A Time-Line for Morphological Changes Following Long-Term Potentiation

The changes in spine morphology after LTP reported in the studies reviewed here are varied and often even contradictory. In fact, basically every possible effect, be it upregulation or downregulation of a certain parameter, has by now been reported: After dentate gyrus LTP, larger spines with shorter necks were found in some studies (Fifkova & Anderson 1981), whereas others report that these parameters are unaffected (Trommald et al 1996). These studies in turn describe increases in spine number (Trommald et al 1996), but other studies are inconsistent with this (Desmond & Levy 1990). In CA1 LTP, similar discrepancies emerge: Some studies report no (or very small) changes in spine morphology or number (Hosokawa et al 1995), whereas others show considerable numbers of new spines (Engert & Bonhoeffer 1999, Toni et al 1999) or filopodia (Maletic-Savatic et al 1999). Moreover, a true assessment of the situation is further complicated by the fact that negative findings are normally not published. Therefore, studies failing to show a relation between LTP and changes in spine morphology are most likely missing from the literature.

There are many reasons for this confusing state of affairs. One is that the changes that occur after conventional, tetanus-induced LTP can only be expected in a subset of spines, and therefore it becomes critical how the potential loci for changes are identified (Engert & Bonhoeffer 1999, Toni et al 1999). Another option is to activate many more synapses by inducing LTP chemically (Hosokawa et al 1995) or even by environmental manipulations (Andersen & Soleng 1998), procedures so different that it is perhaps not surprising that different results have been obtained. Furthermore, the electron-microscopic studies, although best suited to study the minute changes occurring on the level of spines and synapses, have—more than the other studies—the problem that they heavily rely on statistics. In this

respect the advent of two-photon microscopic techniques has brought a substantial improvement in that morphological changes can be observed online, making them not only much more vivid for the human observer but also introducing the element of temporal correlation, which adds considerably to the confidence of a result. It is our feeling then that for this reason some of the recent time-lapse imaging experiments have provided more widely accepted evidence that morphological changes do occur after LTP. The functional consequences of these changes remain, however, the subject of speculation.

We therefore propose a possible sequence of morphological events that accompany LTP. Our proposal, although it tries to integrate as many previous studies as possible, for the reasons stated above, emerges largely from the consideration of more recent data. In order to reflect on the functional consequences of these data we also try to incorporate the biophysical consequences of the observed morphological changes.

Potentially, then, the sequence of events might be as follows (see Figure 1):

1. Initially, within minutes after potentiation, functional changes that are not (or at least not easily) detectable morphologically occur at the potentiated synapse. Such changes include modifications in postsynaptic receptor composition as well as alterations in synaptic release properties as postulated for the early phase of LTP (e.g. Malinow & Tsien 1990, Stevens & Wang 1994, Malenka & Nicoll 1999).
2. Approximately 30 minutes after induction, the first morphological changes become detectable with light or electron microscopy. Spine heads from stimulated spines become larger (Fifkova & Van Harrefeld 1977,

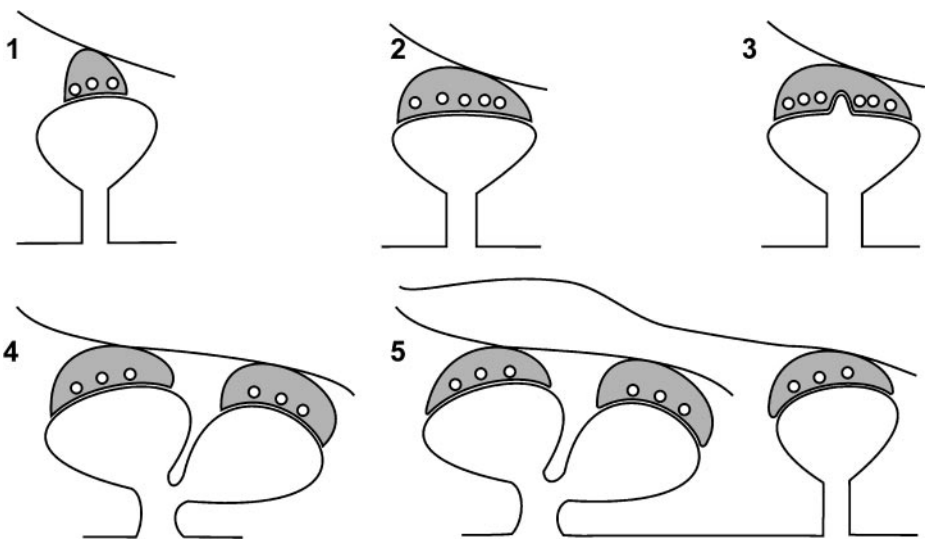

Figure 1 Possible sequence of morphological events after LTP.

Desmond & Levy 1986b) and there is a concomitant increase in synaptic area (Desmond & Levy 1988). Because of a direct relationship between spine volume and synaptic strength, reflected in an increase in the number of postsynaptic receptor and presynaptic docked vesicles (Schikorski & Stevens 1999), this increase could therefore be—at least in part—responsible for the late phase of LTP, as it would provide an enduring structural modification tied to a change in synaptic strength. Besides, enlargement of the spine heads and the widening and shortening of spine necks (Fifkova & Anderson 1981) would have immediate functional consequences bringing about major changes in calcium compartmentalization, thereby influencing the rules for synaptic plasticity (Majewska et al 2000a,b).

3. Synapses that were already large break apart, forming perforated synapses (Peters & Kaiserman-Abramof 1969, Toni et al 1999).
4. Bifurcating spines (Trommald et al 1990) and ultimately even closely associated pairs of spines emanating from one dendrite and touching the same presynaptic element occur (Toni et al 1999). These morphologies could implement the increase in independent synaptic release sites found in some studies of late-phase LTP (Bolshakov & Siegelbaum 1995, Bolshakov et al 1997).
5. Finally, new spines can also form in the vicinity of the activated spines (Engert & Bonhoeffer 1999, Toni et al 1999).

We therefore propose that there is a continuum of morphological events that can occur after the induction of LTP. They range from slight enlargements of synaptic surfaces or spines to the generation of completely new structures. Clearly, in many cases not all steps of the process will be taken, but morphological changes of lesser extent are probably the end-result of synaptic potentiation. In a way, Cajal and Tanzi were both right because we can envision LTP-related changes that require novel neuronal growth as well as those that only involve functional changes in existing connections.

It also is worthwhile to emphasize that although the correlation between LTP and some morphological events such as spinogenesis is well documented (Engert & Bonhoeffer 1999, Toni et al 1999), it is important to take it as such: correlation not necessarily proving causality. Whereas LTP can produce morphological rearrangements, those changes may or may not contribute to the potentiation. It might seem the simplest scenario that new spines contribute to the enhanced efficacy of the observed synapses, yet other possible roles for the new spines are viable, and perhaps even more likely. It is, for instance, possible that the new spines are created as dendritic sites for future plasticity. One might hypothesize that they only contain silent, i.e. purely NMDA, synapses (Isaac et al 1995, Liao et al 1995).

Thus, over the past few years considerable progress has been made in pinpointing morphological changes that correlate with changes in synaptic strength, but

there is still ample room for discovery to understand the true function of these morphological changes.

ACKNOWLEDGMENTS

RY is funded by the National Eye Institute (EY 111787 and 13237) and the NINDS (NSHD 40726). TB is funded by the Max-Planck Gesellschaft. We thank Tim Bliss, Nancy Desmond, Alan Fine, Chip Levy, and Steve Siegelbaum for comments and Chuck Stevens for discussions.

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