Functional and Morphological Plasticity of Dendritic Spines in the Hippocampus

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To my family

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Summary

On CA1 pyramidal neurons, the majority of excitatory synapses are located on dendritic spines. Previous experiments demonstrated that the induction of LTP can modify spine numbers and morphology. However, there was no direct proof if and when the newly grown spines are contacted by a presynaptic terminal and potentially form a functional synapse.

To address this, the extent of colocalization of newly grown spines with antibody staining for either synapsin, a marker of mature presynaptic terminals or GluR2, a subunit of postsynaptic AMPA receptors common at functional synapses was determined. Growth of dendritic spines was induced by extracellular local high frequency stimulation or by using a “chemical LTP” induction protocol.

Overall, the number of spines that colocalized with synapsin puncta seemed to increase with the age of the spine. This indicates that new spines grown upon LTP induction initially lack presynaptic innervation suggesting that new spines do not emerge from pre-existing shaft synapses but protrude towards an existing presynaptic contact. Most of the newly grown spines are GluR2 negative, suggesting that these spines do not contain a fully functional synapse within the first six hours of existence.

Because LTP leads to spine growth, the question was if LTD induces the retraction of previously existing spines. Using two-photon time-lapse microscopy, it was observed that low-frequency stimulation induced NMDA receptor-dependent spine retractions, while theta-burst stimulation led to the formation of new spines, as reported previously. Thus, spines on CA1 pyramidal neurons from organotypic slice cultures can

undergo bidirectional morphological plasticity; spines can be formed and eliminated in an activity-dependent way.

Zusammenfassung

Die große Mehrheit der exzitatorischen Synapsen von Pyramidenzellen der CA1 Region im Hippocampus liegt auf dendritischen Dornen, den sogenannten *spines*. Wie bereits gezeigt wurde, führt LTP (Langzeit Potenzierung; *longterm potentiation*) zu vermehrtem *spine* Wachstum. Bisher konnte jedoch nicht bewiesen werden, dass diese neugebildeten *spines* auch tatsächlich synaptische Kontakte ausbilden.

Zu diesem Zweck wurde die Kolokalisation der neu gewachsenen *spines* mit dem immunohistochemischen Signal für Synapsin, einem Protein das an der aktiven Zone der präsynaptischen Membran vorkommt bzw. mit dem postsynaptischen GluR2, einer Untereinheit des AMPA Rezeptors welche an elektrisch aktiven Synapsen im Hippocampus unabdingbar ist, bestimmt. Die Auslösung von LTP, induziert durch lokale extrazelluläre Stimulation bzw. durch chemische Stimulation aller Synapsen führt zum Auswachsen neuer *spines*.

Offensichtlich kolokalisieren neu gebildete *spines* nicht von Anfang an mit einem Synapsin Punkt und daher einer ausgebildeten Präsynapse, die Wahrscheinlichkeit der Kolokalisation steigt aber mit dem Alter des *spines*. Daraus kann geschlossen werden, dass diese *spines* nicht aus bereits existierenden Synapsen auf dem Dendriten herausgewachsen sind, sondern an neuer Stelle einem bereits bestehenden synaptischen Kontakt entgegenwachsen. Den meisten neuen *spines* fehlt in den ersten sechs Stunden postsynaptisches GluR2 was bedeutet, dass diese *spines* (noch) nicht Teil einer funktionellen Synapse sind.

Da LTP zum Auswachsen neuer dendritischer *spines* führt liegt die Vermutung nahe, dass LTD die Elimination bereits bestehender *spines* bewirkt. Im zweiten Teil der

voliegenden Dissertation konnte erstmals gezeigt werden, dass - je nach Stimulus - neue *spines* gebildet und bestehende *spines* zurückgezogen werden: während LTP zu vermehrtem *spine* Wachstum führt, kommt es nach LTD tatsächlich zu einer Verminderung der spine Zahl. Die Morphologie von CA1 Pyramidenzellen unterliegt damit genau wie die synaptische Stärke einer bidirektionalen Plastizität.

Introduction

General Introduction

The mammalian central nervous system receives, integrates, and processes information about its environment. The output response resulting from processing this information can be modified in response to previous experience, what is generally referred to as learning and memory. Learning influences the neuronal morphology and causes the formation of dendritic spines, structures that are the main carriers of excitatory synapses.

Do these novel spines indeed contain functional synapses? Does the reverse effect, namely the erasure of memory and the depression of synapses lead to pruning of dendritic spines? Despite the numerous studies dealing with synaptogenesis these questions so far remained unresolved. This thesis aims to address these questions in two independent studies.

Synapses

The functional unit of the nervous system is the neuron. The human brain for instance consists of about 100 billion of neurons. Neurons differ in many respects from other cell types. The key neuronal function is the ability to propagate information. The connection between neurons or neurons and other excitable cells where signals are propagated from one cell to another are called synapses, a term Sherrington formulated more than a century ago (Foster, 1897). Two basic forms of synaptic transmission exist, electrical or chemical, depending on how the electrical stimulus is propagated, i.e. either

directly via gap junctions or via neurotransmitters. In the nervous system of vertebrates electrical synapses are rare, but can be found for instance in the retina.

At chemical synapses, electrical activity leads to the release of transmitter from the presynaptic cell into the synaptic cleft. After binding of the neurotransmitter molecules to receptor proteins on the postsynaptic membrane, the postsynaptic cell reconverts this chemical signal into an electrical signal and ensures the propagation of information. Depending on the type of chemical synapses (and the type of neurotransmitter which is released upon electrical stimulation) one distinguishes between inhibitory and excitatory synapses.

Spines

Postsynaptic receptors for excitatory or inhibitory transmission are located at different positions of the neuron. While inhibitory synapses are found predominantly on the cell soma, most of the excitatory synapses, especially in cortical or hippocampal pyramidal neurons are typically located at the tip of dendritic spines, tiny protrusions extending from the dendrite (Harris and Kater, 1994; Nimchimsky et al., 2002). A spine typical for the CA1 region of the hippocampus consists of a narrow neck and a more voluminous head. The total spine volume ranges from 0.004-0.6 μm^3 (Harris and Stevens, 1989). Spines exist in a variety of morphological forms. They are generally classified as thin, stubby, mushroom shaped or branched spines (Jones and Powell, 1969; Harris et al., 1992; Hering and Sheng, 2001). Some authors have added another category, the filopodium, named for its hairlike morphology, which is mostly found early in development (Skoff and Hamburger, 1974). Spines contain the postsynaptic components of the synapse. These postsynaptic structures not only include glutamate receptors (e.g.

NMDA and AMPA receptors) but also receptor binding proteins, signal transduction molecules, and scaffolding proteins anchored in a matrix of cytoskeletal and signaling molecules (Garner et al., 2000; Ziff, 1997; Kennedy, 1997). This meshwork of postsynaptic proteins appears as an electron dense thickening and is therefore called PSD (postsynaptic density). The majority of spines contain one continuous PSD, representing one synaptic contact. Nevertheless, the PSD of some spines appears interrupted and therefore perforated, and it was suggested that these perforated synapses represent an intermediate step during spine division and are thus involved in the process of synaptogenesis (Geinisman et al., 1993).

One possible function of dendritic spines is the increase of the surface area of dendrites and thus the number of possible synapses per dendritic length (Swindale, 1981). Furthermore, spines allow dendrites to reach multiple axons, minimizing the distances from one synapse to the next (Bonhoeffer & Yuste, 2002). In addition, the narrow spine neck restricts the diffusion of molecules into and out of the spine. This diffusional biochemical compartmentalization may help to retain molecules at the synapse, i.e. Ca^{2+} influx upon synaptic stimulation is limited to the stimulated spine and does not affect synapses on neighboring spines (Sabatini et al., 2001; Nimchimsky et al., 2002).

Ramon y Cajal first described dendritic spines more than a century ago. By this time he thought that spines connect axons and dendrites, and he proposed that spines constitute the site of learning and stable long-term memory in central neurons (Ramon y Cajal, 1891, 1893). Soon it was commonly believed that functional changes of excitatory synapses, as they reside on spine heads, are accompanied by morphological changes at the level of single spines or synapses and that regulating this strength of connections is

the cellular basis of learning and memory storage (Ramon y Cajal, 1911; Hebb, 1949; for review see also Segal, 2005).

Morphological plasticity in vivo

Evidence has accumulated that the shape and density of spines may be altered by a range of developmental, pathological, and experimental influences. In vivo, spines undergo a high level of morphological plasticity such as changes in the density or shape during development (Harris, 1999), during the estrus cycle of female rats (Woolley et al., 1996), or during hibernation, when squirrels first lose 40 % of their spines but recover that loss in a few hours after arousal from hibernation (Popov et al., 1992). Furthermore, various learning paradigms cause alterations in spine number and shape (Leuner et al., 2003; Geinisman et al., 2001). Accordingly, neurons from animals raised in a complex environment or trained for specific skills exhibit a more complex arborization of the dendritic trees and a higher density of spine synapses (Greenough et al., 1985; Weiler et al., 1994).

The hippocampus

Different forms of learning and memory require the hippocampus which is therefore extensively used as a model system for studying these phenomena (Becker et al., 1980; Morris et al., 1982; Eichenbaum et al., 1986). The hippocampus is part of the archicortex, the anatomically oldest part of the cerebral cortex and surrounds the thalamus with its longitudinal shape. Like all archicortical structures the hippocampus consists of three cell layers, most importantly the stratum pyramidale which contains mainly pyramidal neurons. The hippocampus is divided into four different regions as

manifested by the morphological differences between the pyramidal neurons (Cornu ammonis: CA1- CA4; Lorente de No, 1934). Each transversal hippocampal slice contains a trisynaptic excitatory pathway: input from the enthorinal cortex is transmitted via the perforant pathway to the dentate gyrus; the mossy fiber pathway connects the dentate gyrus with area CA3; and the Schaffer collateral pathway arises from CA3 pyramidal neurons and terminates on the dendrites of CA1 pyramidal neurons.

Hippocampal slice culture

The monosynaptic Schaffer collateral pathway in the transversally sliced hippocampus allows to specifically manipulate pre- and postsynaptic neurons. Thus, this pathway provides a commonly used model system for studying learning and memory in vitro. Aside from using slices from acute hippocampus preparations, it is possible to cultivate a hippocampal slice under appropriate temperature and medium conditions for weeks and even month (Gähwiler, 1981; Stoppini et al., 1991) thereby gaining organotypic hippocampal slice cultures.

For electrophysiological as well as for imaging studies these organotypic hippocampal slice cultures provide a favorable model system with various advantages compared to acute slices. In acutely sectioned slices the superficial layers contain mainly cellular debris resulting from the slicing procedure while undamaged neurons can only be found in deeper layers of the slice. This side effect of the preparation procedure can be circumvented if the slice is cultivated for a certain time period. During the cultivation the cellular debris on the surface is removed, neuronal processes regenerate, and the culture flattens considerably. Acute slices are several hundred µm thick and are therefore not suitable for imaging studies, particularly when using single photon microscopy. In

contrast, the thickness of organotypic slice cultures is reduced to a few cells in height (~ 50 µm), which facilitates imaging experiments also over extended periods of time.

Compared to dissociated neurons, the organotypic slice cultures offer the unique advantage of a relatively well preserved connectivity of the neuronal network, similar to the in situ organization typical for the hippocampus (Muller et al., 1993; Stoppini et al., 1991, 1993). In addition, pyramidal cells of dissociated cell cultures do not have the typical shape like the apical/basal orientation.

Slice cultures provide a controllable environment with easy access to the cells so that the morphology of individual cells and their protrusions can be monitored easily. Even if neurons from cultures differ from neurons in vivo in respect to some pharmacological and morphological properties - i.e. they have slightly smaller spine density (Papa et al., 1995) the basic rules of cellular interactions and the neuronal organization are comparable to in vivo hippocampi (Frotscher and Gähwiler, 1988; Lendvai et al., 2000; McKinney et al., 1999; Harris et al., 1992). The disadvantages of the slice cultures appear trifling, still there are limitations regarding the artificial growth environment compared to the in vivo situation and regarding the temporal regulation of the developmental program. In terms of synapse formation, promiscuous conditions compared to the in vivo situation have been reported. The neurons in vitro form functional synapses with other neurons regardless whether these cells would normally form synapses in vivo (Vaughn, 1989).

Slice cultures are not ideal regarding various labeling techniques, such as vital dye labeling (i.e. FM dyes) or immunohistochemical stainings on fixed tissue which are often used for single cell cultures. The three dimensional tissue restricts access to less

superficial cells, renders the discrimination of individual staining puncta difficult and displays a high amount of fluorescent background compared to single cell cultures.

Long Term Potentiation (LTP)

Long Term Potentiation (LTP) is an in vitro model of learning and memory. According to the theory of Donald Hebb (1949), who postulated that the regulation of the synaptic strength between two neurons could be the cellular basis for learning and memory, only the coincident activity of the pre- and the postsynaptic cell can lead to changes in synaptic efficiency. Decades later Bliss and Lomo (1973) showed that the magnitude of EPSP´s (excitatory postsynaptic potentials as an indicator for synaptic strength) was potentiated after electrical high-frequency stimulation (HFS). This activity-dependent long lasting increase in synaptic strength was called LTP. That LTP forms the basis of learning and memory is now widely accepted and this process is therefore extensively used to study synaptic plasticity (Bliss and Collingridge, 1993).

Morphological plasticity in vitro

As mentioned above dendritic spines show the remarkable capacity to undergo rapid structural changes, especially following learning paradigms. Could this morphological plasticity possibly represent the basis for learning and memory?

In the last decades, numerous in vitro studies examined the behavior of dendritic spines after various stimulation protocols that elicit LTP. It was assumed that an increase in synaptic strength is accompanied by morphological changes on the level of single spines. In fact, early studies of the effect of LTP on the morphology of dendritic spines showed that tetanization of the perforant pathway leads to an increase in spine volume in

the hippocampal dentate gyrus (van Harreveld and Fifkova, 1973). Soon, the combination of electrophysiological recording and subsequent EM imaging of the potentiated tissue showed that high frequency stimulation (HFS), which produces LTP, led to an increase of synaptic contacts of up to 50 % (Lee et al., 1980). However, the spine volume remained constant in this study. The authors speculated that LTP might be associated with increased innervation of dendritic shafts and an increased number of shaft synapses (see also Yuste and Bonhoeffer, 2001).

Spines are very small structures; therefore they are at the limit of optical resolution of conventional light microscopy. Until recently, it was not possible to study the cellular morphological alterations such as the subtle changes in spine size, length, or number with the high temporal and spatial resolution needed. The development and improvement of new imaging techniques, most notably two-photon microscopy (Two-Photon Laser Scanning Microscopy, TPLSM), allowed time-lapse studies of dendritic structures and helped to investigate morphological changes of spines, that may be associated with LTP (Denk et al., 1990; reviewed in Yuste and Bonhoeffer, 2001). TPLSM overcame the previous problems of bleaching of fluorescent markers and photodynamic damage in living tissue as well as the limited penetration of light used for analysis. Thus, this modern imaging technique provides an ideal tool to observe neuronal morphology over an extended period of time, especially for thick tissue like brain slices or in vivo brain preparations, but also for slice cultures.

Time-lapse confocal imaging could demonstrate that a chemically induced form of LTP, which was expected to potentiate many if not all synapses in an acute hippocampal slice, led to changes in spine length (Hosokawa et al, 1995). In 1999,

several independent studies demonstrated that electrical stimulation (resulting in LTP at Schaffer collateral/CA1 synapses) induced the growth of new postsynaptic spines on CA1 pyramidal neurons. Maletic-Savatic et al. showed that in organotypic hippocampal slice cultures, local stimulation with a strong tetanus, which normally induces LTP, led to the outgrowth of dendritic filopodia-like processes in the proximity of the focal stimulation electrode. The emergence of these protrusions could be blocked by the NMDA receptor antagonist APV which usually blocks LTP (Maletic-Savatic et al., 1999). Similarly, Engert and Bonhoeffer strengthened this correlation between synaptic potentiation and the generation of new dendritic protrusions in slice cultures by TPLSM time-lapse imaging of novel spines grown using a more moderate stimulation (Engert and Bonhoeffer, 1999). In their experiments, potentiation could be restricted to a small region of the postsynaptic cell, by the use of "local superfusion". Applying this technique the synapses outside the superfusion spot were inhibited by a medium containing low extracellular calcium and cadmium, and a small area was superfused with medium containing high calcium, thus allowing synaptic transmission. Upon electrical LTP induction significantly more spines emerged within the superfusion zone than outside.

In the same year, Toni et al. used a combination of electrophysiology and post-imaging electron microscopy to show that LTP led to a two- to threefold transient increase in the ratio of perforated synapses (partioned PSD) versus non-perforated synapses one hour after LTP induction. In addition, the transient emergence of perforated synapses was followed by an increase of multiple synaptic boutons, i.e. an axon terminal that is contacted by at least two spines arising from the same dendrite. Consequently, the

perforated synapses may represent an intermediate step in the process of spine division and synaptogenesis through either a splitting or a budding mechanism (Toni et al., 1999).

More recent work showed that spines can also exhibit more subtle modifications such as changes in their shape after the induction of synaptic plasticity (Matsuzaki et al., 2004). In this study, stimulation led to a transient threefold increase in spine volume after 2-4 minutes, which fell to a ~20-30 % increase in spine volume 20-40 minutes after stimulation. Therefore, LTP induction causes an increase in the allover synaptic surface of a cell, either by increasing the number of synaptic contacts or by enlarging the synaptic surface of individual existing spines.

Together, all these experiments demonstrated that LTP is correlated with persistent alterations in spine number and shape. These alterations could provide a mechanism for memory storage, by strengthening and refining of particular synaptic connections or building new synapses on newly grown spines in response to previous experience.

Proposal of the sequence of morphological events

Various experiments from the past years demonstrated a link between synaptic plasticity and the growth of new spines. In light of these and various studies, the following proposal of a possible temporal sequence of morphological events on the dendrite that accompany LTP can be presented (Yuste and Bonhoeffer, 2004; and references therein):

1. Immediately after stimulation, no obvious morphological changes occur. On the molecular level modifications in the receptor compositions as well as alterations in synaptic release probability have been reported (Malinow &Tsien 1990).

2. Subsequently, first morphological changes become visible, i.e. larger spine heads (Fifkova and van Harreveld, 1977; Matsuzaki et al., 2004) and/or an increase in the synaptic area (Desmond and Levi, 1988). Ultrastructurally, an increase in the number of postsynaptic receptors and presynaptic docked vesicles can be observed (Schikorski et al., 1999).

3. As a result of the enlargement of the synaptic area the large synapses seem to break apart and form perforated synapses (Toni et al., 1999).

4. The perforated synapses segregate further and form bifurcated spines (Trommald et al., 1990), so that pairs of spines (maybe emerged from the same "motherspine") are innervated by the same presynaptic terminal (Toni et al., 1999).

5. Finally, the formation of a novel spine is complete (Engert and Bonhoeffer, 1999; Toni et al., 1999).

Morphological changes following LTP: the open questions

All these studies, demonstrating the (temporal) correlation of synaptic plasticity and the formation of novel spines failed to address two main issues. First, none of these studies has actually verified that these new spines in fact contribute to the enhanced synaptic depolarization in response to the afferent stimulation, the hallmark of LTP. Second, the involvement of the presynaptic terminal remains unclear. It has not been demonstrated so far that spines and filopodia, grown upon LTP induction in hippocampal slice cultures are in contact with a presynaptic terminal and are or become part of functional synapses. Recent experiments have shown that in dissociated neurons spines grown de novo upon enhanced network activity are likely to be innervated by a presynaptic terminal, labeled with FM dye (Goldin et al., 2001). These results indicate

that in dissociated cell cultures long-term functional changes are indeed correlated not only with morphological modification but also with the formation of new synaptic contacts. So far there was no direct demonstration in organotypic slice cultures that activity-dependent spine growth leads to the formation of functional synapses.

One possibility to answer this question would be to demonstrate that newly grown spines are associated with FM labeled presynaptic terminals, thereby assessing the relation between the fate of a spine and its association with an active terminal, as previously described for dissociated cell cultures (Goldin et al., 2001). This is rather difficult in the slice culture because of the high amount of fluorescent background due to the difficulty of washing out the dye out of the three-dimensional tissue. Consequently it is impossible to discriminate individual puncta.

Simultaneous time-lapse imaging of spine growth as well as of the presynaptic terminal could also demonstrate if the novel spine is associated with a synaptic contact. Unfortunately, this experiment appears to be rather difficult. Most transfection or staining techniques result in labeling of only a subset of neurons. While this sparse labeling is crucial for discriminating individual cellular components in the dense meshwork of cells in slice cultures, the problem is that one cannot be sure that the appropriate presynaptic terminal to the imaged postsynaptic spine is indeed labeled. Therefore, the lack of an innervating presynaptic terminal would not necessarily prove the absence of functional innervation, but possibly the lack of efficient staining.

Another possible approach to address the question of presynaptic innervation of newly grown spines is to do postimaging immunohistochemical characterization of previously imaged spines grown upon LTP induction and to characterize their molecular

composition concerning the pre- and postsynaptic site. The establishment of this technique was one of the two main goals of this dissertation.

Pruning of spines

In the developing nervous system, the refinement and modulation of neuronal circuitry is not only driven by synapse formation but also by disassembly of previously functional synapses and thereby pruning of spines. Thus, there is an initial overproduction and later elimination and reduction of synapses early in postnatal development (Rakic et al., 1986). Furthermore, pruning of spines is a natural process even in the matured nervous system. It can be observed for instance during the estrus cycle of female rats (Woolley et al., 1996) or in hibernating animals.

Bidirectional morphological plasticity

Regarding the synaptic efficacy of hippocampal synapses bidirectional effects have been reported, namely long-term potentiation (LTP) after high-frequency stimulation (HFS) and long-term depression (LTD) after low-frequency stimulation (LFS) (Bliss and Lomo, 1973; Dudek and Bear, 1992; Mulkey and Malenka, 1992; Liu et al., 2004). As mentioned above, several experiments in hippocampal slice cultures demonstrated that strengthening of synapses by inducing LTP results in significant structural changes on dendrites such as the outgrowth of dendritic protrusions.

Whether, conversely the reduction in size or the complete retraction of spines or filopodia correlated with LTD-inducing stimulation has not been explored. The retraction of spines following LTD would suggest that bidirectional functional synaptic plasticity,

LTP and LTD, may be closely associated with bidirectional morphological plasticity. The second goal of this dissertation was to monitor morphological spine changes during LTD.

Summary of aims

There is strong evidence showing that synaptic strength and the morphology of spines are plastic depending on previous experience. Previous studies could demonstrate a correlation between LTP, the cellular model for learning in vitro, and the formation of new spines. In line with these previous results two main questions remain unresolved:

First, it has not been demonstrated so far that novel spines contain functional synapses. Although the presence of new functional synaptic contacts would not answer the question whether these new spines in fact contribute to the enhanced synaptic activity following the afferent stimulation, the knowledge about if and when a spine receives presynaptic innervation would help to understand better the cellular mechanisms induced by learning and memory.

Second, since the potentiation of synapses during LTP results in morphological changes such as the growth of new spines or filopodia, the question whether in reverse synaptic depression during LTD leads to the opposite effect, namely the reduction in size or the complete retraction of spines was not answered for a long time.

The present study aimed to address both of these questions in two separate studies. First, an immunohistochemical staining technique was established to characterize newly grown spines of known age in respect to colocalization with presynaptic terminals and to colocalization with postsynaptic AMPA receptors. Second, the effect of low-frequency stimulation normally used to induce LTD on the morphology of dendritic spines was examined. Using time-lapse TPLSM, the dendritic structure was imaged before and after extracellular electrical stimulation and morphological dendritic changes were assessed by counting the numbers of spines that grew or retracted.

Chapter 1: Immunohistochemical characterization of activity-dependent spinogenesis in hippocampal neurons

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Summary

On CA1 pyramidal neurons, the majority of excitatory synaptic contacts are located on dendritic spines. Previous experiments demonstrated that induction of LTP leads to morphological changes on these neurons, such as the formation of new spines. However, it is an open question if and when the newly grown spines are contacted by a presynaptic terminal and form a functional synapse. To address this, we determined the extent of colocalization of newly grown spines with antibody staining for synapsin, a marker of mature presynaptic terminals. Further we tested the immunoreactivity of newly grown spines to GluR2, a subunit of postsynaptic AMPA receptors common to functional synapses. Using two-photon time-lapse microscopy and high resolution confocal microscopy, we imaged dendritic spines of CA1 pyramidal GFP-positive neurons in organotypic slice cultures of Thy1-GFP mice. Selective growth of dendritic spines was induced by extracellular local high frequency stimulation or by using a "chemical LTP" induction protocol. We acquired high-resolution image stacks of dendritic stretches continuously for up to six hours. This allowed us to narrowly confine the age of the newly born spines prior to fixation and processing for immunohistochemistry. A considerable fraction of the newly born spines colocalized with synapsin staining, indicating that these spines are often in the immediate vicinity of presynaptic terminals. The number of spines that colocalized with synapsin puncta seemed to increase with the age of the spine suggesting that novel spines initially lack presynaptic partners, while at later stages new spines abut presynaptic specializations more often. In contrast, most of the newly grown spines are GluR2 negative, suggesting that these spines do not contain a fully functional synapse.

Taken together, our data suggest that new spines grown upon LTP induction do not emerge from pre-existing shaft synapses but protrude towards an existing presynaptic specialization, mostly not forming functional synapses within the first six hours of existence.

Introduction

Dendritic spines are the main sites of synaptic input onto neurons as they receive excitatory inputs from presynaptic cells, especially in cortical or hippocampal pyramidal neurons (Nimchimsky et al., 2002). In the CNS, they account for 90 % of excitatory synaptic transmission in the brain (Harris and Kater, 1994).

In vivo, spines undergo a high level of morphological plasticity such as changes in the density or shape during development (Harris 1999), during the estrus cycle (Wooley et al., 1996), during the course of hibernation (Popov et al., 1992), or following various learning paradigms (Leuner et al., 2003; Geinisman et al., 2001).

Furthermore, in vitro experiments corroborated the variability of the dendritic spine density. So is the shape and number of dendritic spines also influenced in an activity-dependent manner: early electron microscopy studies showed that tetanization of the perforant pathway, which usually induces LTP, had an effect on the morphology of dendritic spines such as increased spine number and volume after stimulation (Van Harrefeld and Fifkova, 1975).

More recent experiments using time-lapse two-photon microscopy confirmed these findings and demonstrated that stimulation of the Schaffer collaterals following a brief LTP-producing stimulus led to an increased spine number in CA1 pyramidal neurons as well as increased filopodia numbers (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999) or to an enlargement of spine size (Matsuzaki et al., 2004). However, none of these studies could show a correlation between this evoked spinogenesis and the formation of a functional synapse on these newly grown spines. Recent experiments with dissociated neurons showed that spines grown de novo upon

enhanced network activity are likely to be innervated by a presynaptic terminal, labeled with FM dye (Goldin et al., 2001). These results indicate that at least in dissociated cell cultures long-term functional changes are indeed correlated not only with morphological modifications but also with the formation of new synaptic contacts.

Similarly to the uncertainty of the innervation of new spines in organotypic slice cultures, the pre- and postsynaptic orchestration of synapse formation is also not yet clear: different models of spinogenesis exist, one assuming that the dendritic spine emerges independently of the axonal terminal while another suggests that a presynaptic terminal induces the formation of new spines (reviewed in Yuste and Bonhoeffer, 2004). The existence of postsynaptic markers prior to the contact to a presynaptic axon terminal would suggest that new spines already contain a postsynaptic specialization. Likewise, colocalization with presynaptic markers before colocalizing with postsynaptic markers could suggest that the presynaptic terminal somehow promotes the formation of the postsynaptic specialization. Experiments in dissociated cell culture showed that the assembly of the postsynaptic density (PSD) occurs at a later timepoint than the formation of a presynaptic active zone (Friedman et al., 2000). However, there may be a potentially different time-course of events in dissociated cell cultures compared to the more complex situation of organotypic slice cultures.

In this study, we established a high resolution staining procedure to be able to characterize spines immunohistochemically. This allowed us to determine if newly grown spines are contacted by mature presynaptic terminals. Moreover, we tested for the presence of the AMPA receptor subunit GluR2 in newly grown spines, using it as a marker for functional and active synapses.

Before immunohistochemistry, we monitored activity-dependent morphological plasticity of the dendritic structures in organotypic slice cultures of Thy1-GFP mice. Green fluorescent protein (GFP) positive CA1 neurons were imaged either with two-photon laser-scanning microscopy (TPLSM) or with high-resolution confocal microscopy. Selective growth of dendritic spines was induced by extracellular local stimulation with a theta-burst protocol or by using a “chemical LTP” induction protocol.

Our results of the posthoc immunohistochemical staining against pre- and postsynaptic markers on previously imaged and new spines of defined age allowed us to suggest the following model which connects spinogenesis with synaptogenesis: Spines grown de novo upon LTP induction do not emerge from functional dendritic shaft synapses indicated by the absence of colocalization with the active zone marker synapsin in the first hours of existence. The colocalization of new spines, older that two hours, with presynaptic terminals suggests that the spines tend to grow towards a synaptic contact formed by a mature spine and an axon terminal in the vicinity. The new spine itself does not form a functional synaptic contact with the axon terminal in the first hours of existence, since most of the novel spines lack postsynaptic GluR2. It is possible that the existing contact splits at a later timepoint and the new spine forms a synapse itself.

Results

Time-lapse imaging

One possible morphological change after synaptic plasticity is the formation of new dendritic spines. We wanted to address the question if and when these newly grown spines are innervated by a presynaptic terminal and form a functional synapse. Time-lapse observation and subsequent immunohistochemical characterization of the newly grown spines should help to characterize their synaptic identity.

Either high-resolution two-photon imaging or confocal imaging was used to follow the morphological changes of dendritic spines of GFP-positive CA1 pyramidal neurons in organotypic hippocampal slices. Imaging at 30 or 60 minutes intervals allowed a faithful tracking of the fate and age of individual dendritic spines.

To induce activity-dependent spine growth, two different stimulation protocols were used, either by local electrical stimulation of the Schaffer collateral pathway or by bath application of a "chemical LTP" induction protocol. Following these stimulation procedures, we reproduced earlier data showing that LTP like stimulation (both electrically or chemically induced) leads to an activity-dependent growth of new spines (Nägerl, Eberhorn et al., 2004; Engert and Bonhoeffer, 1999; Toni et al., 1999; Hosokawa et al., 1995). Figure 1A shows an overview of fluorescent CA1 pyramidal neurons. The imaged neurons all had characteristic CA1 pyramidal cell morphologies. Figure 1C shows in higher magnification a stretch of dendrite corresponding to the dashed outlined region in Figure 1A, which was imaged over time. The blue circle marks the fluorescent tip of the electrode. The same dendritic stretch at a later timepoint after electrical stimulation is shown in Figure 1D, a newly grown spine is marked by the

arrow. Thus, using these induction protocols, the formation of new spines could be reliably induced and faithfully tracked over time using combined LTP-producing stimulation and high-resolution imaging techniques.

Posthoc immunohistochemistry

To gain insight into the molecular composition of the de novo grown spines, the slice cultures were fixed immediately after the last imaging timepoint, processed for immunohistochemistry, and subsequently imaged by confocal microscopy. By carefully handling and processing the slices, the overall morphology of the neurons was well maintained (compare Figure 1A and 1B after fixation) so that previously imaged individual spines could be readily reidentified in the immunohistochemically stained preparation. The fixed sample of Figure 1D is shown in Figure 1E, illustrating that, despite lower image quality after immunohistochemical processing, the reidentification and analysis of individual spines is possible. Thus, even near the limit of optical resolution, we can reidentify structures as small as a spine in dense three-dimensional tissue.

Three-dimensional immunohistochemistry in organotypic slice cultures

We established a high-resolution immunohistochemical staining technique to determine the colocalization of individual spiny protrusions with presynaptic terminals and to analyze the molecular composition of single dendritic spines in the three-dimensional hippocampal slice culture. Synapsin was used as a ubiquitous marker of presynaptic terminals; postsynaptically, the immunoreactivity against the GluR2 subunit of the AMPA receptor was assayed.

To quantitatively asses the staining efficiency, the number of spines that colocalized with synapsin (Figure 2A) or GluR2 (Figure 2C) puncta were counted on CA1 pyramidal neurons in an organotypic slice culture. The criterion for colocalization was defined such that the fluorescent signal of the immunoreactive punctum had to overlap or abut with that of the spine in at least one plane of the imaged stack. Colocalization of reidentified spines and puncta was analyzed in single imaging planes rather than the Maximum Intensity Projection that were used for display in the Figures.

Of the mature spines, i.e. spines which were continuously present throughout the time-lapse recording, 84 % showed a clear colocalization with presynaptic terminals (two examples are shown in Figure 2B). Figure 2A demonstrates that only 5 % of all mature spines did definitely not colocalize with a presynaptic terminal, while for the remaining 11 % the colocalization was not clear. A case was classified as “uncertainly colocalized” when the colocalization did not fulfill our criterion of clear colocalization, i.e. when the fluorescent signal of the spine and the immunohistochemical signal were close but not abutting.

For the immunostainings against GluR2 as a marker for electrically conducting and thereby functional hippocampal synapses, the amount of colocalization was significantly lower. Figure 2C shows that 44 % of mature spines clearly contained GluR2 positive puncta while 33 % of all spines seemed to lack postsynaptic GluR2. Figure 2D shows an example of a spine clearly not colocalized with the GluR2 punctum in the vicinity. The immunopositive staining signal was not overlapping with the spine in any image plane and therefore did not fulfill the criterion of a clear colocalization (for overview of this example see Figure 2E). For GluR2, the number of unclear cases was

with 23 % relatively high, partly due to a weaker and less crisp staining pattern compared to the anti-synapsin labeling which makes it more difficult to compare the colocalization between the spine and the immunohistochemical staining signal.

Double immunostaining for synapsin and GluR2 on the same spine was also possible. The highlighted spine in Figure 2F is an example of a spine, which clearly colocalized with GluR2 (red) (Figure 2G, for GluR2 staining pattern alone see Figure 2H), as well as for the presynaptic marker synapsin (blue). Thus, postimaging immunhistochemical staining in organotypic slice cultures can help to unravel the molecular composition of individual spines and assess their innervation by presynaptic terminals.

Newly born spines often contact presynaptic terminals

To answer the question if and when new spines become part of functional synapses, we combined three-dimensional time-lapse imaging of dendritic stretches of CA1 pyramidal neurons with activity-dependent induction of spine growth to generate de novo grown spines of well defined age. After processing these samples for immunohistochemistry against pre- or postsynaptic markers (synapsin or GluR2, respectively) we reidentified the previously imaged neurons and spines in confocal image stacks and determined their colocalization with these markers. The TPLSM images of Figures 3A and 3B illustrate how individual dendritic spines grow in an activity-dependent manner; time values indicate the time before (- min) after and after (+ min) local stimulation. Figure 3C shows the same spine as above after fixation, immunohistochemistry and volume rendering from confocal image stacks. The age of the spine was 90 minutes. The analysis after immunolabeling with an anti-synapsin antibody

(Figure 3D) showed that this spine did clearly not colocalize with a presynaptic terminal. Clear colocalization is seen in Figures 3E to 3H in which a 210 minutes old spine was in close proximity to an anti-synapsin punctum and thus to a presynaptic terminal. Supplemental Figure 1A shows further four examples of spines that clearly colocalized with a presynaptic terminal marked by positive anti-synapsin staining. All newly grown spines (at an age from 60 minutes to five hours) were located close to a presynaptic terminal. Thus, we can state about the presynaptic innervation of spines that are up to six hours old.

Quantitative and qualitative measurement of the time-course of spine-α-synapsin colocalization

16 de novo grown spines of defined age were reidentified after fixation and immunohistochemical staining against the presynaptic marker synapsin. A summary of the extend of colocalization is shown in the histogram of Figure 4A, red squares indicate that a spine clearly colocalized with a synapsin punctum, while grey squares indicate no colocalization. Not every reidentified spine was in the vicinity of a discernible synapsin punctum, as the Maximum Intensity projections in supplemental Figure 1B illustrate. As evident from Figure 4A and 4B, there appears to be a trend that older spines (i.e. older than two hours) were contacted by a presynaptic terminal more frequently than young spines (60 minutes or younger).

This trend is supported by pooled data from control experiments using low-frequency stimulation (LFS; 900 pulses at 1Hz) in the presence of the NMDA receptor blocker APV. In these experiments, a few spines grew spontaneously, yet there was a clear tendency of older spines being more likely colocalized with synapsin puncta.

Supplemental Figure 2A shows a total 21 of de novo grown spines (pooled data from activity-dependent spine growth, plus five spontaneously grown spines from control experiments). The histogram displays the time-course of synapsin colocalization with newly born and reidentified spines and suggests that colocalization increased with the age of the spine. Supplemental Figure 2B depicts the likelihood of presynaptic innervation of newly emerged spines and illustrates the above mentioned tendency. Because new spines older than two hours are more likely to be contacted by a presynaptic terminal than younger spines, there appears to be a slow physiological maturation process that could eventually lead to the formation of a functional synapse.

Reidentified newly grown spines mostly lack postsynaptic specializations

Colocalization of a spine with a presynaptic marker like synapsin is a strong argument for the spine being part of a functional synapse. On the other hand it is possible that the spine is located in close proximity of a presynaptic specialization without being synaptically innervated. To further investigate whether a spine indeed is part of an electrically conducting and therefore fully functional synapse, the newly grown spines were also tested for their immunoreactivity to the GluR2 subunit of the AMPA receptor.

Figure 5A and 5B show a newly grown spine after local electrical stimulation (time in minutes before (- min) and after (+ min) stimulus). The same spine after fixation and immunohistochemical processing is depicted as a Maximum Intensity Projection in Figure 5C. Even if this spine shows clear colocalization with anti-synapsin (Figure 5D) there is no clear colocalization with a discernible anti-GluR2 punctum (corresponding spine in Figure 5E). Figures 5F-5J illustrate the unique case of a de novo grown spine at the age of 90 minutes that clearly stained for the GluR2 subunit. Figures 5F and 5G

demonstrate the activity-dependent spine growth (time in minutes before (- min) and after (+ min) local stimulation), Figure 5H shows the same spine after fixation and immunohistochemical processing, Figures 5I and 5J show the corresponding anti-GluR2 staining. The green channel was omitted in Figure 5I to allow for better visualization of the GluR2 punctum. The posthoc immunohistochemical staining for GluR2 on previously imaged spines showed that hardly any new spine was immunopositive for GluR2 (N=1 spine was immunopositive for GluR2). Therefore it is likely to assume that new spines are not part of functional synapses.

Quantitative and qualitative measurement of the time-course of spine-GluR2 colocalization

So far, four de novo induced spines were reidentified and immunohistochemically characterized for the presence of GluR2 on the postsynaptic site, as depicted in Figure 4C. Unlike the data for the anti-synapsin staining, older spontaneously emerging spines from control experiments with APV and low frequency stimulation (LFS) did not have an increased probability of colocalizing with GluR2 puncta. This suggest that spines older than two hours are not more likely to form functional synapses even if they tend to colocalize with existing synaptic contacts in the vicinity. In supplementary Figure 2C nine newly grown spines between one and six hours of age were plotted (pooled data). Only one spine out of these nine showed clear colocalization with a GluR2 punctum. This suggests that unlike the majority of mature spines most of the new spines do not contain functional synapses on their tips.

Discussion

Using post-imaging high resolution immunohistochemistry, we established a new approach to determine the molecular composition of newly grown single spines in three dimensional hippocampal slice cultures. With time-lapse two-photon or confocal microscopy to image GFP-positive pyramidal neurons we repeated previous studies (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Nägerl, Eberhorn et al., 2004) and induced rapid spine growth in an activity-dependent manner. Subsequent immunohistochemical stainings against pre- and postsynaptic markers demonstrated that most of these de novo generated spines do not contain active synapses within the first six hours of existence even if they seem to grow towards an existing presynaptic terminal.

So far there was no direct demonstration that activity-dependent spine growth leads to the formation of functional synapses. EM studies have shown that newly generated spines, albeit not by an activity-dependent paradigm, had the appearance of ultrastructurally mature synapses (Trachtenberg, 2002).

There are other possible approaches regarding time-lapse studies of the development of individual synaptic sites to help understand if new spines make functional synapses. First, one could fluorescently label the presynaptic terminal with specific styryl dyes, e.g. with FM dyes, by expressing fluorescently tagged presynaptic markers or dye labeling the whole presynaptic neuron. Together with a differently labeled postsynaptic neuron one could simultaneously monitor the time course of both pre- and postsynaptic events underlying synaptic plasticity. Despite their attractiveness and their applicability in single cell cultures, all these labeling techniques have some drawbacks for three dimensional tissues such as organotypic slice cultures. Since these cultures are

relatively thick (up to a few cell layers) the staining of presynaptic terminals i.e. using FM dyes makes it hard to discriminate between individual puncta and leads to a high amount of unspecifically labeled background. In addition, all these methods do not guarantee a 100 % staining of all neurons in the region of interest, but only a subset of neurons are transfected or dye labeled. To determine the presynaptic innervation of a new spine, one must be sure that the appropriate presynaptic terminal is indeed labeled.

We decided to use postimaging immunohistochemistry to analyze single newly grown spines which were tracked over time. One advantage is that immunohistochemical labeling with anti-synapsin should in principle mark all possible presynaptic terminals. In dissociated cultured neurons, immunohistochemistry has been used extensively to characterize the molecular orchestration of synaptic assembly with great detail. Unfortunately, this staining technique is much less suitable in three dimensional slice cultures. Because the slice cultures are thick and dense compared to sectioned brain slices or dissociated cell cultures, the standard antibody staining protocol was modified by substantially increasing the incubation times to allow for full penetration of the antibody into the tissue. Good results were obtained by incubating the cultures with the primary antibody for 7 to 10 days, but additional protocol modifications were also necessary (see methods). With the modified protocol a global, but specific labeling of antigens was achieved even in deeper sections of the slice culture. High-resolution confocal imaging subsequently allowed immunohistochemical characterization of a previously imaged spine with high precision deep within the slice culture.

Immunohistochemical staining against the presynaptic marker synapsin in hippocampal slice cultures revealed a clear colocalization of mature CA1 spines with

presynaptic terminals in 84 % of all mature spines. This value is in excellent agreement to what has been described in the literature, as 88 % of dendritic spines of CA1 pyramidal neurons in organotypic slice cultures were closely apposed to presynaptic terminals (Richards et al., 2005). This technique is therefore an ideal method for analyzing the colocalization of a spine with a presynaptic terminal. With our approach, only 5 % of all mature spines were clearly not located in the vicinity of any presynaptic punctum. The difference of 11 %, which we consider "uncertain colocalization" is due to our stringent criterion for colocalization: In the high resolution confocal image stacks, the fluorescent signal of both the spine and the immunohistochemical stain had to be overlapping or at least abut in one or more image planes (z distance ~ 400 nm). Potentially, the extent of presynaptic colocalization with mature CA1 spines may therefore be a slight underestimation of the actual value.

The results were different in stainings against the postsynaptic marker and subunit of the AMPA receptor GluR2. Here, 44 % of the mature spines were clearly colocalized with GluR2 puncta, while 33 % were clearly not. This could be explained by the possibility that indeed a higher percentage of spines in organotypic slice cultures do not bare a functional synapse even if the majority of mature spines contact a presynaptic terminal (see above). On the other hand it is also possible that the lower percentage of colocalization between mature spines and GluR2 signal and the higher percentage of uncertain colocalization (23%) is due to technical reasons. Since the GluR2 staining was not as complete and not as crisp as the synapsin staining, in which puncta were more defined and less fuzzy (compare Figure 2) it was difficult to determine true colocalization according to our stringent criterion for colocalization (see above) and the percentage of

clear colocalization is potentially an underestimation. In addition, the amount of postsynaptic GluR2 perhaps was not sufficient to always produce an unambiguous staining signal which would explain the high value of uncertain colocalization.

The aim of this study was to determine if and when newly grown spines become part of functional synapses. Synaptic plasticity, either triggered by electrical or chemical LTP, generated de novo grown spines of well defined age. Doing postimaging immunohistochemistry against presynaptic synapsin or postsynaptic GluR2, we determined the colocalization of the previously imaged spines with these markers. 16 spines, grown de novo in an activity-induced manner between 30 minutes and six hours of age were tested for their colocalization with synapsin, a marker for mature presynaptic terminals. It was shown that older spines (i.e. older than two hours) were contacted by a presynaptic terminal more frequently than young spines (60 minutes or younger). This trend is supported by the finding that also spontaneously grown spines (N=5) older than two hours are more like to be colocalized with synapsin puncta then younger spines. In mature spines (older than six hours; see above) the colocalization with the presynaptic marker culminates in a colocalization of 84 %.

Apparently new spines colocalize at some point with presynaptic synapsin. The question remains whether this colocalization to an active presynaptic terminal indeed resembles the formation of a functional synapse between the newly grown spine and a presynaptic terminal or whether the new spine emerges in close proximity to an existing presynaptic specialization. To answer this question previously imaged spines were tested for their immunoreactivity to postsynaptic GluR2, a subunit of the APMA receptor and a marker for functional and electrically conducting synapses. Surprisingly, only one new

spine (out of four new spines grown in an activity dependent manner) was immunopositive for GluR2 while the remaining cases clearly showed no colocalization with GluR2 staining (in addition, all five spontaneously emerged spines from control experiments lacked postsynaptic GluR2). Since the colocalization rate of GluR2 on mature spines reached a level of 44 % one would expect a significantly higher percentage of colocalization upon the newly grown spines. The almost complete absence of postsynaptic GluR2 upon newly grown spines therefore suggests that - unlike the majority of mature spines - most of the new spines do not contain functional synapses and do not participate in synaptic transmission within the first six hours of existence.

Postimaging electron microscopy studies to look for functional synapses on newly grown spines help to interpret our data (Nägerl et al., 2005, in preparation). Here, electrical stimulation led to activity-dependent induction of spines. These newly grown spines did not show any of the structures associated with functional synapses during the first 8 hours of existence such as a postsynaptic density, a synaptic cleft or a bouton associated with a synaptic specialization. Interestingly, however, the majority of new spines were in contact with presynaptic terminals, which formed synapses with other spines. These findings go very well in line with our results. It is possible that the colocalization between newly grown spines and presynaptic synapsin in the absence of GluR2 colocalization mark spines which contact synapses between presynaptic terminals and other postsynaptic specializations in the vicinity without forming a functional synapse itself.

These data lead to the following model, depicted in Figure 6: Since the likelihood for a spine to colocalize with a presynaptic terminal increases with its age there appears a

slow physiological maturation process of synapse formation. The absence of synapsin colocalization in the first two hours of existence indicates that new spines emerge de novo, i.e. not from a preexisting shaft synapse. After two hours of existence, a higher fraction of newly grown spines contacts presynaptic specializations, possibly the new spines grow towards an existing synaptic contact, formed by the synapsin-positive axon terminal and a mature spine in the vicinity. Unlike mature spines most of the new spines do not contain postsynaptic GluR2 in the first six hours of existence and do therefore not participate in synaptic transmission, even if they contact a presynaptic specialization. This supports the assumption that new spines do not contain fully functional synapses initially after emergence. The insertion of postsynaptic GluR2 might happen at a later timepoint and the maturation process to a fully functional and conducting synapse may require more time. Further tests with older de novo grown spines (older than six hours) will help to address the question if and when new spines become finally part of a fully functional synapse.

We speculate that after a few hours of spine presence, the abutting presynaptic terminal could divide and form two separate synaptic contacts, one with the mature previously existing spine and one with the newly grown spine. This possibility could explain previous results showing that LTP induction elevates the proportion of multiple synaptic boutons in which two spines arising from the same dendrite contact the same presynaptic bouton (Toni et al., 1999).

It is possible that new spines go through a transient state of silent synapses containing only NMDA receptors before these spines become part of fully functional, non-silent synapses where AMPA receptors are postsynaptically inserted. Evidence

accumulated that LTP promotes the insertion of new AMPA receptors in postsynaptically silent synapses and that this conversion from silent into functional synapse is one way to increase the synaptic efficacy. To follow that possibility additional immunohistochemical stainings against the NMDA receptor are necessary.

Methods

Organotypic hippocampal cultures.

Hippocampal slices from postnatal day 5-7 transgenic mice (thy1-GFP-mice, courtesy of J. Sanes, Washington University, St Louis, MO) were prepared as previously described (Gähwiler, 1981; Nägerl et al., 2004) and were incubated for up to 3 weeks after preparation.

Recording solutions.

For electrical stimulation, cultures were transferred to a recording chamber and continuously perfused with carbogenated ACSF containing NaCl, 126 mM; KCl, 2.5 mM; $CaCl_2$, 2.8 mM; $MgCl_2$, 0.5 mM; glucose, 10 mM; NaH_2PO_4, 1.25 mM; $NaHCO_3$, 26 mM; glycine, 0.05 mM; pyruvate, 1 mM (pH 7.4). In one control experiment, 50 µM APV (D-2-amino-5-phosphonovaleric acid) was bath applied for 45 minutes (30 minutes before and throughout low-frequency stimulation (LFS), consisting of 900 pulses delivered at 1 Hz).

For the chemical LTP induction protocol, slice cultures were first continuously perfused with NaCl, 137 mM; KCl, 2.7 mM; $CaCl_2$, 2.8 mM; $MgCl_2$, 20 mM; glucose, 5.6 mM; NaH_2PO_4, 0.4 mM; $NaHCO_3$, 11.6 mM. For inducing chemical LTP, the perfusion solution was then replaced for 15 minutes with NaCl, 124 mM; KCl, 5 mM; $CaCl_2$, 5 mM; $MgCl_2$, 0.1 mM; glucose, 10 mM; $NaHCO_3$, 24 mM; NH_2PO_4, 1.25 mM; TEA (Tetraethylammonium), 25 mM (Adapted from Hosokawa et al., 1995). Subsequently, the perfusion was switched back to the original solution.

In both cases, the temperature was maintained at 35º C.

Electrophysiology.

Patch pipettes were used for electrical stimulation. They were filled with 3 M NaCl and additionally contained 10 mM of the fluorescent dye calcein immobilized in agar for better visualization of the electrode in the tissue. A chlorided silver wire was used to pass brief current pulses (0.2 ms) of 15-30 µA from a stimulus isolator (WPI, Berlin, Germany) through the patch pipette. The stimulus protocol used for LTP was a theta-burst-stimulation (TBS) consisting of 5 trains (200 ms inter-train interval) of 6 pulses delivered at 100 Hz, repeated 5 times every 10 sec. The tip of the stimulating electrode was positioned in close proximity of the stretch of apical dendrite that was selected for time-lapse imaging. The minimal distance between the tip of the electrode and the dendrite was kept between 10-20 µm. We confined stimulation to the apical dendrites of CA pyramidal neurons, because of their known ability to undergo activity-dependent morphological plasticity (Engert and Bonhoeffer, 1999; Nägerl et al., 2004).

Microscopy.

Time-lapse two-photon laser-scanning microscopy was used to image the morphology of GFP-positive CA1 pyramidal neurons and to follow potential changes in dendritic morphology. The red excitation light (λ = 840 nm) from a 5 W Mira-Verdi laser system (Coherent, Santa Clara, CA) was routed through a Fluoview 200 scanner (Olympus, Hamburg, Germany), a suitable dichroic mirror (LOT Oriel, Darmstadt, Germany) and a 40×, 1.2 NA water immersion objective (Zeiss, Oberkochen, Germany) mounted on an inverted IX70 microscope (Olympus). The power of the excitation light could be adjusted continuously by an acousto-optical modulator (Polytec, Waldbronn, Germany) and its average value at the objective was set to 10-20 mW. The fluorescence

was detected by an external photomultiplier tube (R6357, Hamamatsu, Herrsching, Germany). Image acquisition and online analysis was performed by the Fluoview software (Olympus) with an image resolution of 115 nm/pixel in x-y. A piezo-electrical actuator (Physik Instrumente, Karlsruhe, Germany) was used to move the objective in the z axis ($\Delta z = 0.4$ µm). Images were Kalman-averaged over three frames, acquisition speed was 3.3 sec/scan.

In experiments in which spine growth was elicited by the induction of chemical LTP, images were recorded with a Leica SP2 laser scanning confocal fluorescence microscope (Leica, Heidelberg, Germany). Images were taken with a 63 x glycerol objective, Numerical Aperture at a resolution of around 60 – 100 nm per pixel.

In both cases, the imaged stack was about 20 µm thick and encompassed the entire dendritic tree of interest. To follow the dendritic morphology, stacks were acquired every 30 or 60 minutes, with two to three stacks recorded before the stimulation and up to ten stacks after induction of LTP. Stacks were carefully aligned to reduce sample drift that occurred during the experiment.

Image analysis I.

4D (x,y,z,t) image stacks were processed and analyzed using Imaris 4 software (Bitplane, Zürich, Switzerland). Individual stacks were spatially filtered by an edge-preserving algorithm, re-scaled, and baseline subtracted. To facilitate overview and for display in the Figures, the 3D stacks were volume rendered as 2D images using the blending projection view in Imaris. All image analysis was done by visual inspection of the individual image sections as well as the rendered 2D projections of the image stacks.

Posthoc-immunohistochemistry and laser-scanning confocal microscopy.

Right after the time-lapse imaging, the slice cultures were fixed with 4 % PFA over night. After rinsing in PB and incubation in blocking and permeabilization buffer (1.5 % horse serum, 0.1 % BSA, 0.4 % Triton X-100 in PB) over night (4° C), cultures were incubated for 7 – 10 days (4°C) in one of the following primary antibodies in 1.5 % horse serum, 0.4 % Triton X-100 in PB: anti-synapsin (polyclonal, 1:500) or anti-GluR2 (monoclonal, 1:500; both Chemicon, Temecula, CA). After rinsing in PB and incubation with secondary antibodies in 0.4 % Triton X-100 in PB (either goat anti-rabbit-cy3, goat anti-mouse-cy3, goat anti-rabbit-alexa 633, or goat anti-mouse-alexa 633, all 1: 500) for 1-2 days (4° C) slice cultures were mounted in Gelmount (biomeda, Foster City, CA) and imaged with a laser-scanning confocal microscope (SP2, Leica). Confocal images of the previously imaged regions of CA1 pyramidal neurons were acquired using a 20 x or a 63 x glycerol objective at a resolution of 40 – 60 nm per pixel. Dual or triple channel imaging of GFP/cy3/alexa 633 staining combinations was sequentially recorded.

Image analysis II.

After fixation and immunohistochemical staining procedures, the de novo grown spines were reidentified by image analysis with maximum intensity projections using the Leica SP2 software and Metamorph 6.1 (Universal Imaging Inc., Downingtown, USA). The immunohistochemical labeling was assessed in single sections of confocal image stacks to ensure that potential colocalizations were not an artifact of the maximum intensity projections. 3D image stacks were volume rendered as 2D images in Imaris (see above). For the images depicting the spine and the corresponding antibody staining, maximum intensity projections were produced by using only sections with significant

fluorescent signal of the spine of interest to reduce the background. Quantification of the number of colocalized spines with either anti-synapsin or GluR2 puncta was done in single planes of 3D image stacks.

Figures

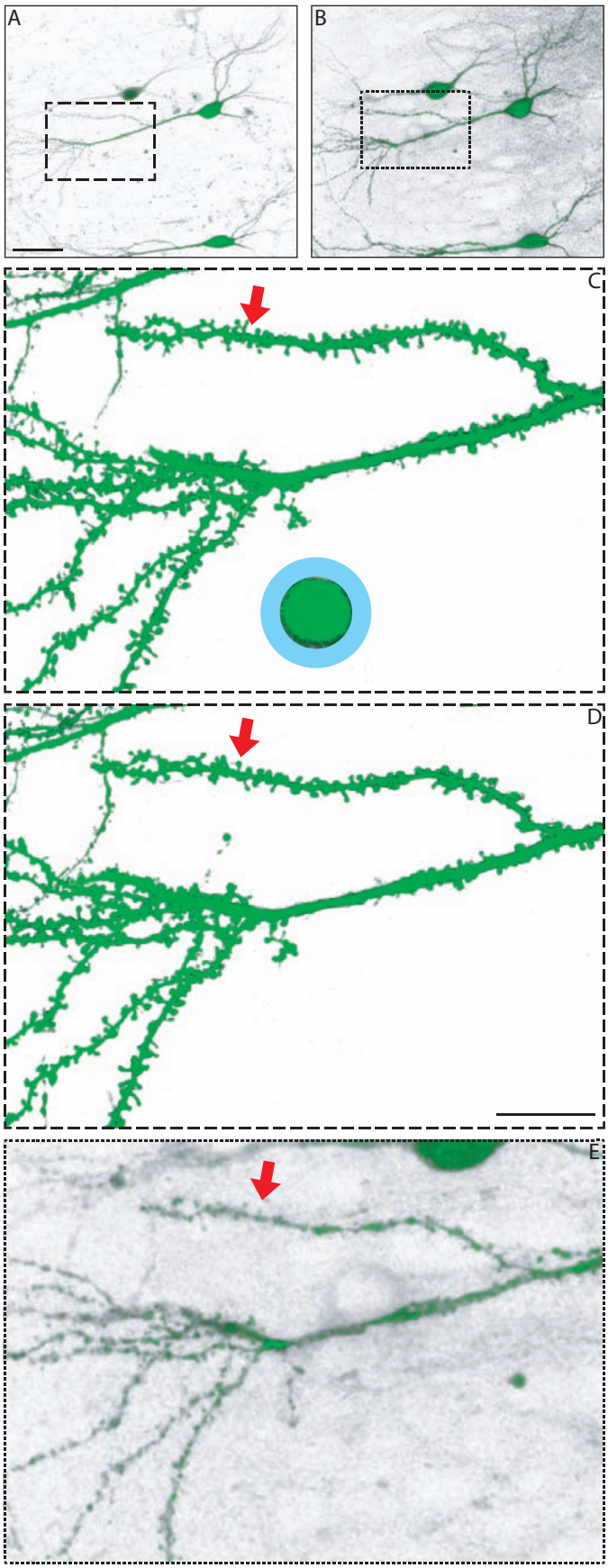
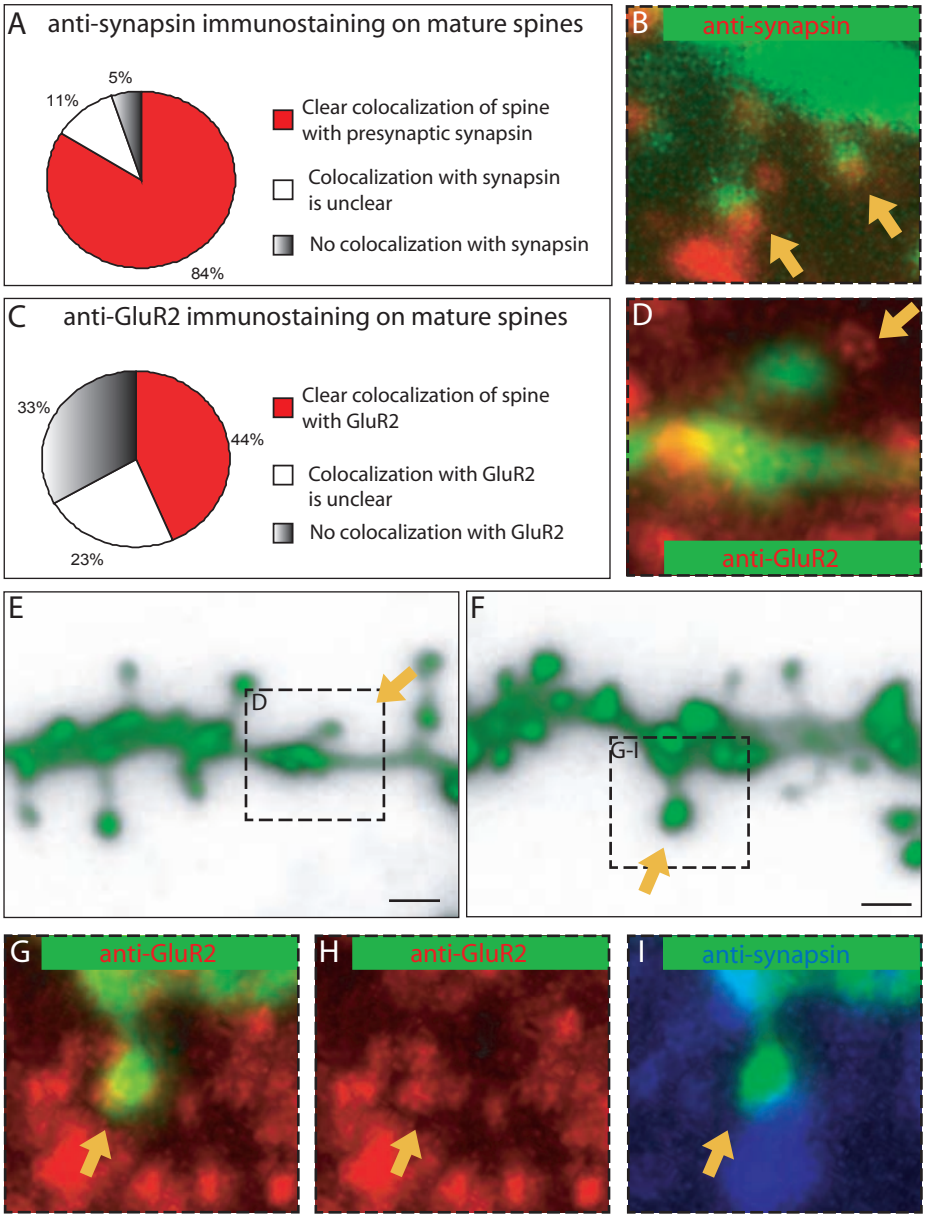


Figure 1: Eberhorn et al. (2005)



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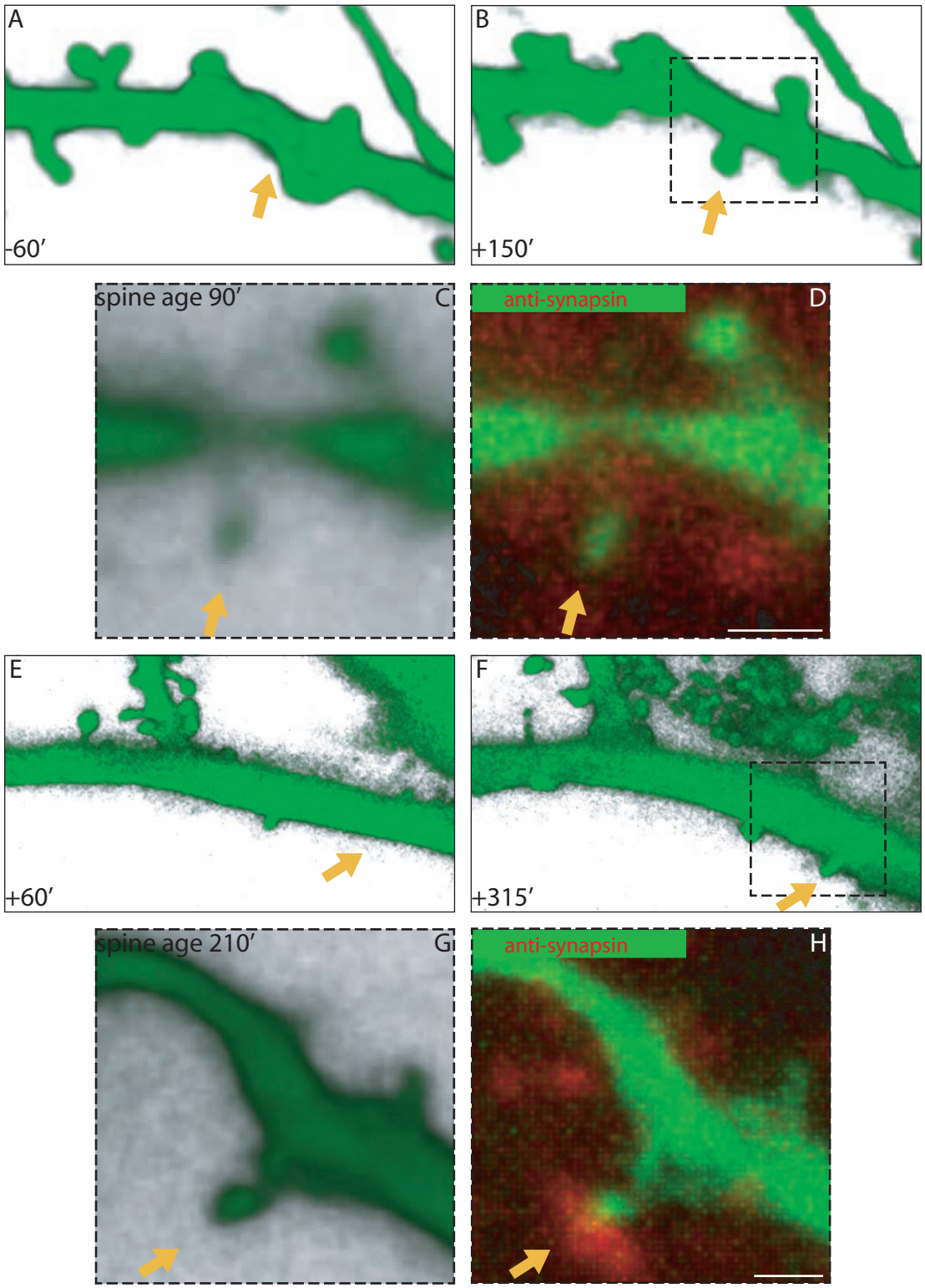

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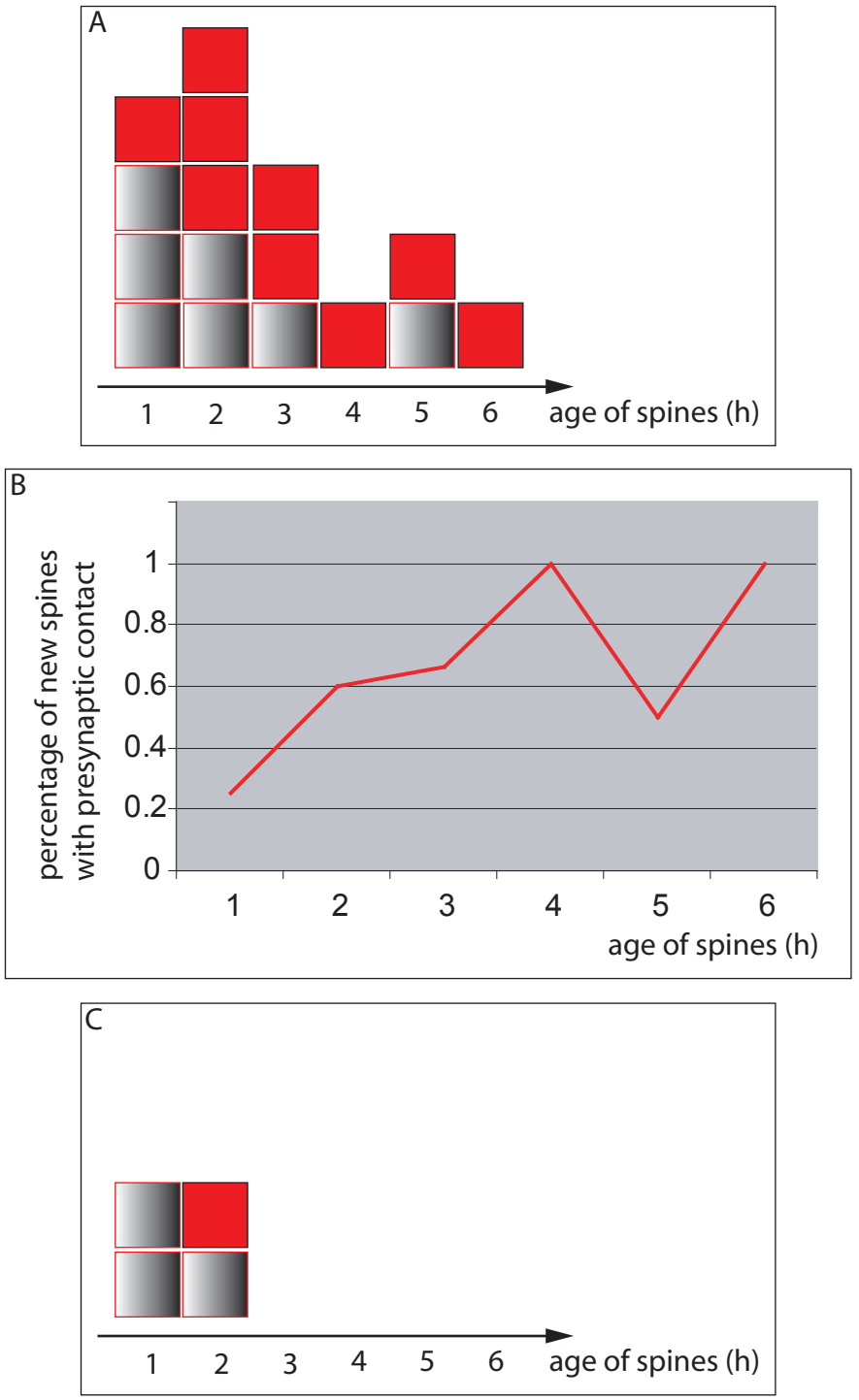

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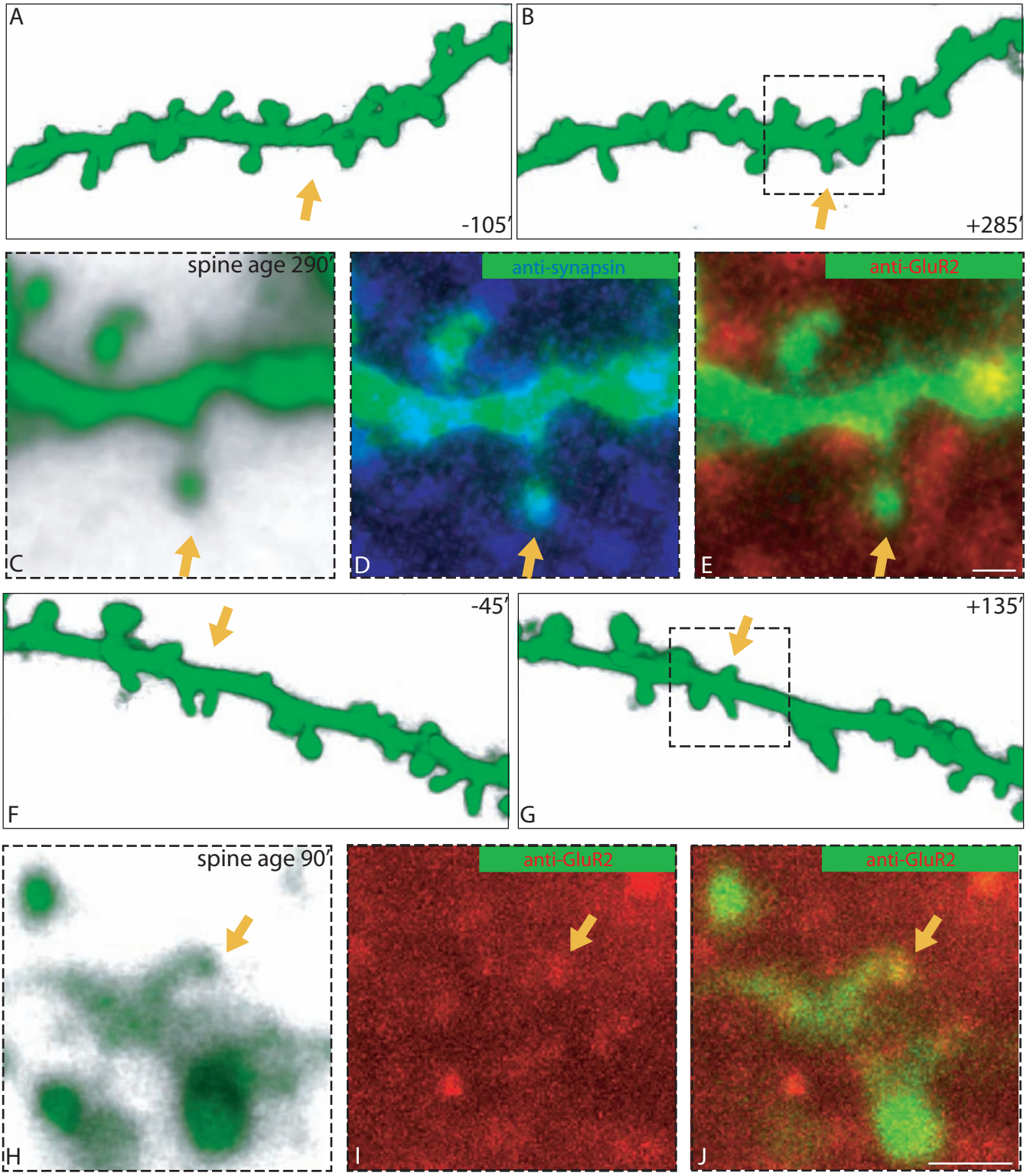

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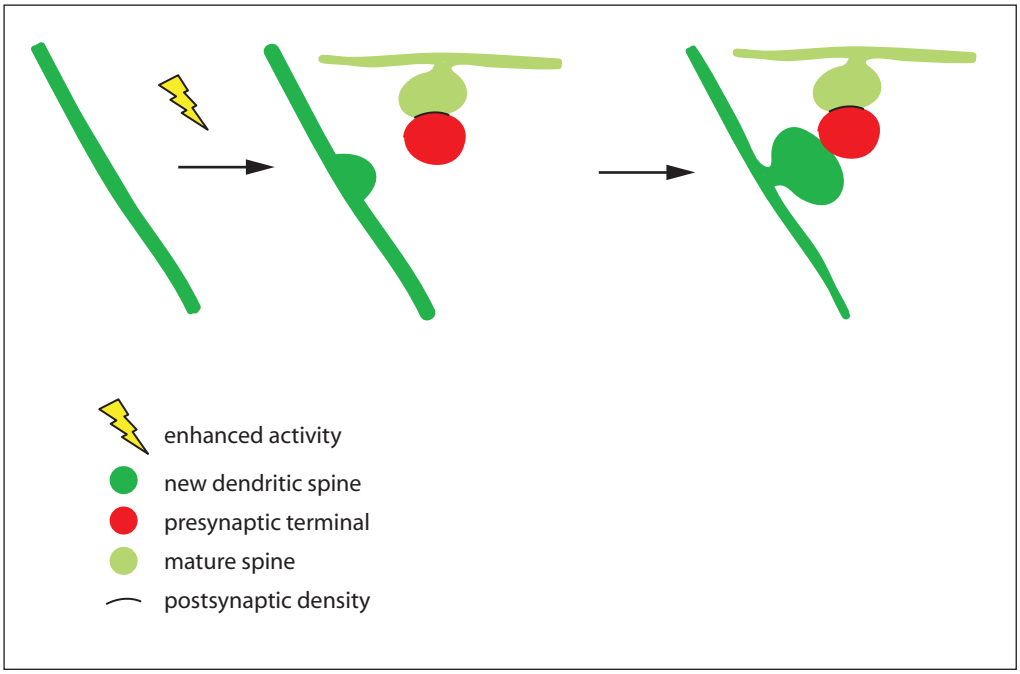

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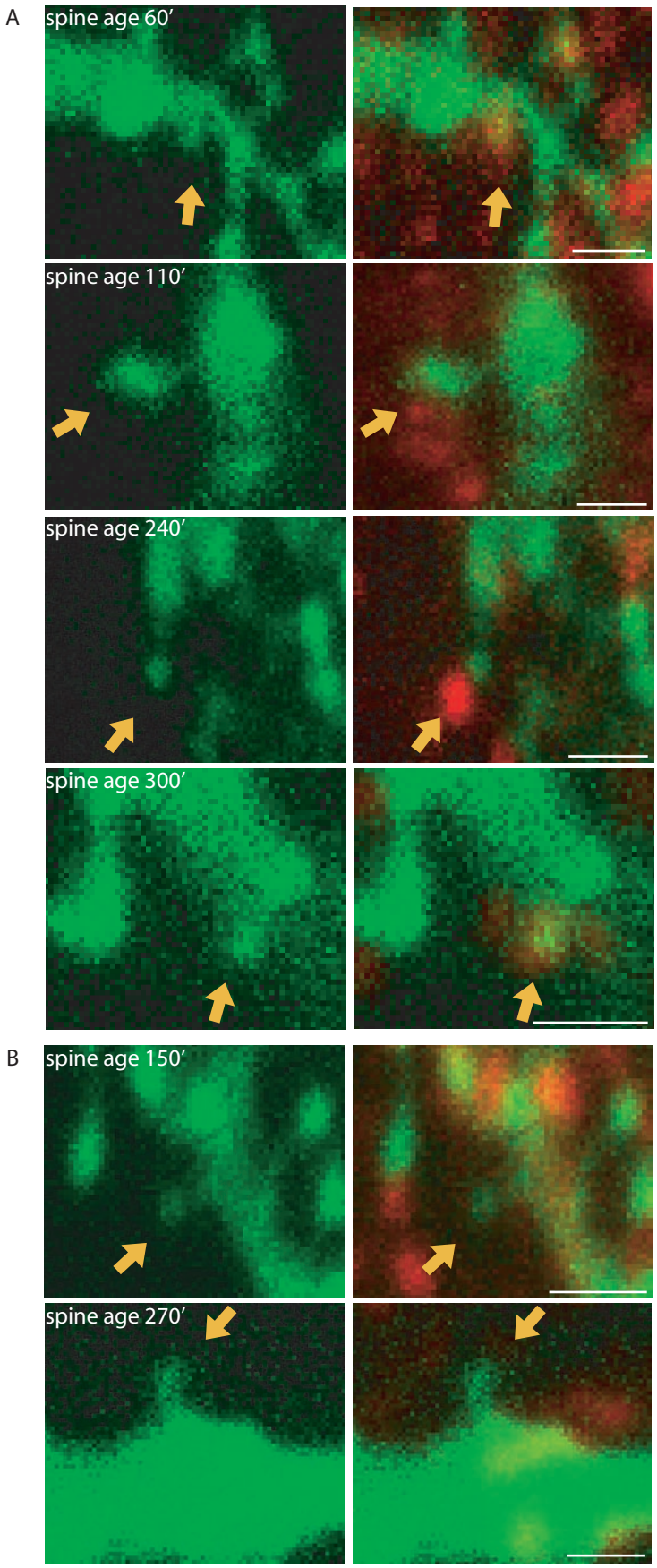
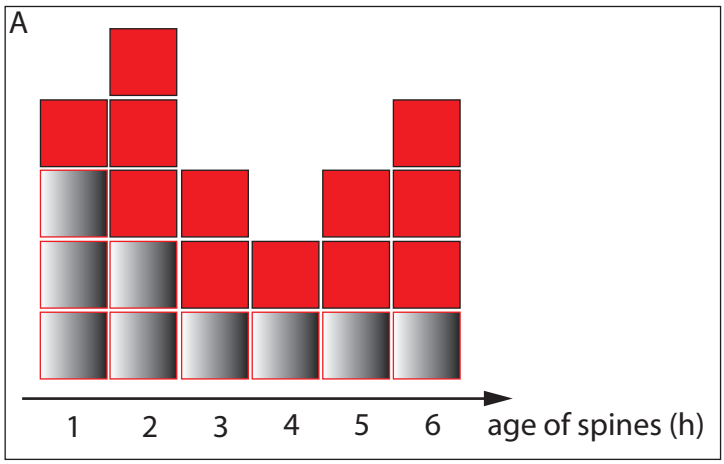
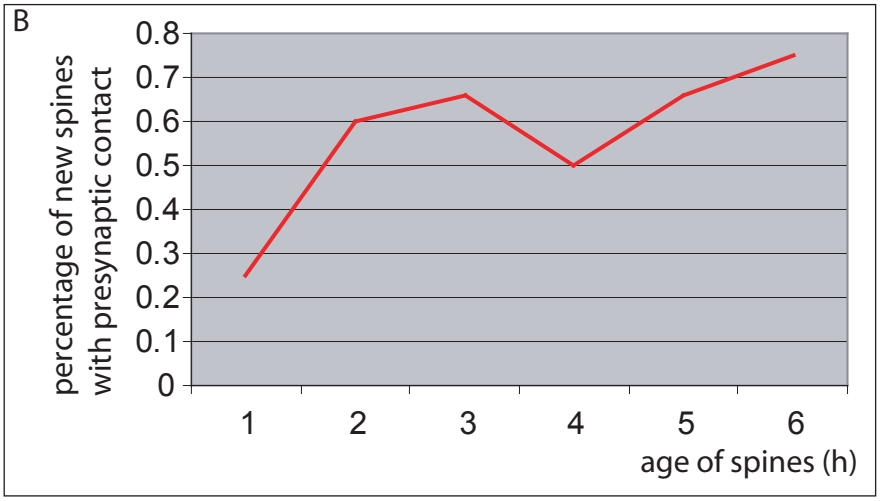
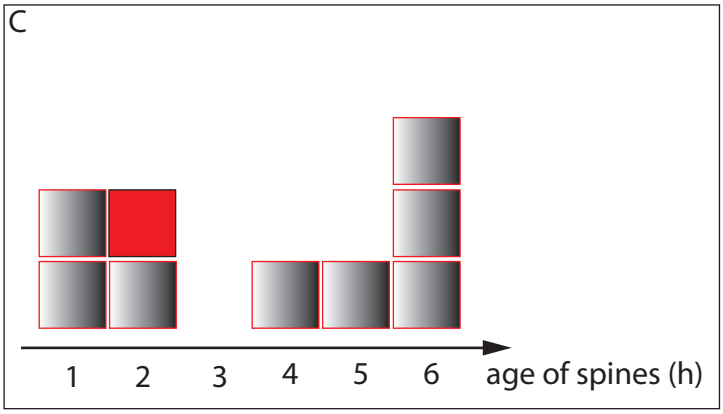


supplemental figure 1: Eberhorn et al. (2005)







supplemental figure 2: Eberhorn et al. (2005)

Figure Legends

Figure 1: Time-lapse imaging of dendritic fine structure and their reidentification after fixation and immunohistochemistry.

A Overview of GFP-positive CA1 pyramidal neurons prior to fixation (TPLSM) and **B** after fixation and processing for immunohistochemistry (MIP of confocal stack). Scale bar, 50 µm. Magnified areas (dashed outlined region) of **A** are depicted before **C** and after **D** extracellular stimulation. Blue circle marks the fluorescent tip of the electrode. Arrow points to a de novo grown spine and its locus after fixation. Images were processed by Kalman averaging, filtering, volume rendering, and background subtraction. **E** shows the same dendritic stretch after fixation and immunohistochemistry. Scale bar, 20 µm.

Figure 2: Quantification of colocalization of mature spines and immunohistochemical staining.

A Quantification of anti-synapsin staining show 84 % colocalization of mature spines with presynaptic terminals. Total of 173 spines, 4 different experiments. **B** displays two examples of clear colocalization between spines and synapsin puncta.

C-I GluR2 immunostaining marked sites of potential synapses. **C** Quantification of GluR2 positive staining on mature spines showed a clear colocalization in 44 % of all spines while 33 % of mature spines seemed to lack postsynaptic GluR2. Total of 145 spines, 4 different experiments. **D** shows an example of a spine with no colocalization with GluR2 punctum, **E** overview of the same dendritic stretch. **G-I** show magnified area of **F** and highlight an example of a mature spine with discrete colocalization to a red

GluR2 punctum (**G** and **H**). Scale bar, 1 µm. Note that the same spine is also positive for the presynaptic marker synapsin (**I**, in blue), suggesting a presumable synaptic contact.

Figure 3: Reidentified newly grown spines often contact presynaptic terminals.

A and **B** TPLSM images of dendritic stretches before (- min) and after (+ min) TBS. Arrows indicate de novo grown spine. **C** and **D** corresponding dendrite after fixation and immunohistochemistry indicating that the newly grown spine (age = 90 min) was not contacted by a presynaptic terminal. **E** and **F** TPLSM images of dendritic stretches after (+ min) TBS; arrows indicate de novo grown spine. **G** and **H** show that the newly formed spine, age 210 min, clearly colocalized with a presynaptic synapsin punctum. Scale bars, 1 µm.

Figure 4: Histogram and time-course of the colocalization between activity induced newly grown spines and immunopositive puncta.

A Synapsin colocalization with newly grown spines. Young spines (age ~ 1 hour) are more likely to lack a presynaptic partner than older spines (age > 3 hours) which were contacted by a presynaptic terminal more often. **B** shows that only one newly grown spine out of 4 displayed GluR2 positive colocalization.

Figure 5: Reidentified newly grown spines mostly lack postsynaptic specializations.

A and **B** TPLSM images of stretches of dendrites before (- min) and after (+ min) electrical stimulation. Arrows mark a de novo grown spine. **C** shows the corresponding spine after fixation. Immunostaining against synapsin (blue puncta in **D**) and GluR2 (red puncta in **E**) show that after 290 minutes of existence, the spine was clearly contacted by an presynaptic terminal but lacked GluR2 positive staining. **F** and **G** example of a de

novo created spine (age at fixation 90 minutes), which clearly colocalized with a GluR2 positive punctum **H-J**. Scale bars, 1 μm.

Figure 6: Model for synaptogenesis on de novo grown spines.

Synaptic plasticity induced either by local electrical stimulation or chemical LTP leads to selective spine growth on CA1 pyramidal neurons. Our current model assumes that de novo built spines tend to contact an existing synapse without being innervated by the respective presynaptic terminal. Potentially, the newly grown spine may form a functional contact with this terminal at later stages.

Supplemental Figure 1: Examples of activity induced newly grown spines and their extend of colocalization with anti-synapsin stainings.

A Four examples of three different experiments showing cases of clear colocalization between activity-induced spines and presynaptic synapsin staining (time indicates length of spine existence after de novo growth). Note that the colocalization in the 110 minutes case was indeed lateral, but clearly overlapping and therefore fulfilling our criteria of true colocalization. **B** Two cases in which the newly grown spine was not in the vicinity of any discernible synapsin puncta (time indicates age of spine in minutes). Scale bars, 1 μm.

Supplemental Figure 2: Histogram and time-course of the colocalization between de novo grown spines and immunopositive puncta; combined results of different experimental conditions.

A Histogram and time-course of synapsin colocalization with newly grown spines. Young spines (< 2 hours lifetime) seemed to be mostly devoid of presynaptic

partners while older spines (> 5 hours lifetime) appeared to be innervated by a presynaptic terminal more often. **B** shows the percentage of clearly colocalized newly born spines out of all reidentified spines. Data were pooled from experiments with activity-dependent induction of spine growth in addition to data from control experiments using APV and LFS. **C** shows that only one newly grown spine out of 9 displayed GluR2 positive staining.

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Chapter 2: Bidirectional Activity-Dependent Morphological Plasticity in Hippocampal Neurons

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Summary

Dendritic spines on pyramidal neurons receive the vast majority of excitatory input and are considered electro-biochemical processing units, integrating and compartmentalizing synaptic input. Following synaptic plasticity, spines can undergo morphological plasticity which possibly forms the structural basis for long-term changes in neuronal circuitry. Here, we demonstrate that spines on CA1 pyramidal neurons from organotypic slice cultures show bidirectional activity-dependent morphological plasticity. Using two-photon time-lapse microscopy, we observed that low-frequency stimulation induced NMDA receptor-dependent spine retractions, whereas theta-burst stimulation led to the formation of new spines. Moreover, without stimulation the number of spine retractions was on the same order of magnitude as the stimulus-induced spine gain or loss. Finally, we found that the ability of neurons to eliminate spines in an activity-dependent manner depended on developmental age. Taken together, our data show that hippocampal neurons can undergo bidirectional morphological plasticity; spines are formed and eliminated in an activity-dependent way.

Introduction

The ability to undergo activity-dependent changes in synaptic strength is a hallmark of many neurons in the mammalian central nervous system. Such synaptic plasticity has been intensely studied in the hippocampus, and it is hypothesized that changes in functional connectivity underlie cognitive functions such as learning and memory (Bliss and Collingridge, 1993). While there is ample evidence that synapses in the hippocampus can undergo bidirectional changes of their efficacy (Bliss and Lomo, 1973; Dudek and Bear, 1992; Mulkey and Malenka, 1992; Liu et al., 2004), the physiological and, in particular, the morphological alterations that accompany synaptic long-term potentiation (LTP) and long-term depression (LTD) are only partly understood.

Recent improvements in time-lapse imaging techniques have made it possible to observe changes of dendritic morphology and synaptic function at the level of single synapses in response to plasticity-inducing synaptic activation (reviewed by Yuste and Bonhoeffer, 2001; Emptage et al., 2003). These new technologies have made it possible to test the long-held hypothesis (Cajal, 1911; Hebb, 1949) that functional changes of synapses are accompanied by morphological changes at the level of single spines or synapses. More precisely, it was shown that electrical stimulation resulting in the enhancement of Schaffer collateral/CA1 synapses induces the growth of new postsynaptic spines or filopodia on CA1 pyramidal neurons (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999). Recent work shows that spines can also exhibit more subtle modifications such as changes in their shape after synaptic plasticity (Matsuzaki et al., 2004). These experiments demonstrated that LTP at single

synapses is correlated with persistent spine volume increases, a possibility raised many years ago (van Harreveld and Fifkova, 1975).

While several reports demonstrated that strengthening of synapses results in structural changes on dendrites such as growth of new spines or filopodia, evidence for the opposite effect, namely the reduction in size or the complete retraction of spines or filopodia in response to LTD-inducing stimulation, has not been reported so far.

Here, we further explored the ability of hippocampal CA1 pyramidal neurons to exhibit electrical stimulation-induced morphological plasticity, by time-lapse imaging of the dendritic structure of green fluorescent protein (GFP) positive cells with two-photon laser-scanning microscopy (TPLSM). We used two different stimulation protocols, theta-burst stimulation (TBS; Larson et al., 1986) and low-frequency stimulation (LFS; Dudek and Bear, 1992; Mulkey and Malenka, 1992), which are classically used to induce LTP or LTD, respectively, at the Schaffer collateral/CA1 synapses. We show that – in keeping with earlier results – an LTP-inducing stimulus leads to the growth of new spines. Importantly, we now also demonstrate that low-frequency stimulation results in morphological plasticity, by a clear and statistically significant loss of spines. In addition to these changes, we also find a constant stimulus-independent loss of spines, which we think can be explained physiologically rather than by 'run-down' of the slice culture. Finally, we explore the age-dependence of activity-dependent morphological plasticity and observe that neurons from 4-8 week-old slice cultures show significantly less LFS-induced and stimulus-independent spine loss than younger ones. This suggests that the stability of spines and their resistance to activity-dependent morphological plasticity increases with the developmental age of the tissue.

Our results provide for the first time a link between a LTD-inducing stimulus and spine loss, suggesting that the bidirectional functional synaptic plasticity, LTP and LTD, may be closely associated with bidirectional morphological plasticity.

Results

We used time-lapse TPLSM to image the dendritic morphology of GFP-positive CA1 pyramidal neurons in organotypic hippocampal slices prepared from transgenic mice. Imaging at 30 minute intervals, we determined the time-course of dendritic spines that appeared and disappeared following electrical stimulation of Schaffer collaterals using two different stimulation protocols. Figure 1A shows fluorescent CA1 pyramidal neurons, the red rectangle indicating a typical region of interest on the apical dendrite, containing the fluorescent tip of the electrode (green circle) used for extracellular stimulation. Figure 1B shows a raw image of a stretch of dendrite, and Figure 1C shows the same image after processing, illustrating the improvement in image quality achieved by Kalman-averaging, filtering, and volume rendering.

Theta-burst stimulation induces spine growth

Initially, we wanted to confirm that TBS, which is classically associated with NMDA-receptor-dependent LTP at the Schaffer collateral synapses, is effective at inducing new spines on CA1 pyramidal neurons. Using local field stimulation, we found that dendritic spine growth could indeed be induced by a stimulating electrode positioned in the vicinity of a dendrite. Figures 2A and 2B show representative images from different time series of volume-rendered image stacks before and after TBS stimulation, illustrating the morphological changes we observed. Figure 2D shows the quantitative

analysis of fourteen such experiments with the number of new spines per 100 μm of dendrite plotted against time (solid black circles). As a control, the number of new spines that appeared without any electrical stimulation is plotted as solid red squares. To test whether TBS led to a significant increase in spine number beyond that attributable to baseline spinogenesis, we compared the total numbers of newly grown spines after TBS and under unstimulated control conditions. Three hours after TBS, the number of new spines per 100 μm of dendrite was 1.01 ± 0.17 spines (n = 6) compared to a baseline value of 0.2 ± 0.15 (n = 8) (p = 0.0043; two-tailed t-test). The stimulus-induced spine growth peaks (as indicated by the steepest part of the curve) at about two hours after the stimulation, and levels out at about three hours. Together, these data are in agreement and in support of earlier studies showing that LTP-like stimulation can result in the generation of new spines (Engert and Bonhoeffer, 1999; Toni et al., 1999; Jourdain et al., 2003).

We also quantified the population of spines that first emerged and then disappeared, termed 'transient spines'. No differences (see Figure 2D, grey symbols and lines) were detected in the rates at which transient spines were created during the unstimulated condition and after TBS. Most transient spines were present only at one data point indicating that they are relatively short-lived.

Theta-burst stimulation does not induce spine retraction

All these earlier studies had focused on the question whether a LTP-like stimulus leads to the formation of new spines. We also investigated the possibility that TBS, in addition to producing new spines, also leads to retraction of existing spines. The open symbols in Figure 2D show the number of spine retractions for the stimulated and

unstimulated control cases (negative values). The number of spines (per 100 µm of dendrite) that retracted over a three hour time window after TBS (0.73 ± 0.29; n = 6) was statistically not distinguishable from the number of the unstimulated control condition (0.9 ± 0.23; n = 8; p = 0.65, two-tailed t-test). Figure 2C shows an example of two spines that retracted spontaneously in such an experiment. Interestingly, the number of retractions of the unstimulated control condition is not much smaller than the number of spines grown after TBS (1.01 ± 0.17, n = 6), so that there is hardly any net gain in the number of spines under these conditions (p = 0.42, two-tailed t-test).

Low- frequency stimulation induces spine retraction

Next, we tested whether a stimulus known to be effective in inducing LTD leads to the retraction of spines on CA1 pyramidal neurons. Apart from using a low-frequency stimulus (LFS; 900 pulses at 1Hz) the experiments were identical to the TBS experiments described above. We first confirmed electrophysiologically that local stimulation with LFS indeed caused LTD (data not shown). Representative examples of the morphological effect of this stimulus are shown in Figures 3A and B, illustrating that spine retractions can occur in an all-or-none fashion. Furthermore, only some spines retracted, while other spines in the neighborhood remained unaffected for many hours. Blocking NMDA receptors by application of 50 µM APV at the time of LFS completely prevented this effect. The open symbols in Figure 3C show the time courses of the spine loss for the conditions of LFS, LFS with 50 µM APV, and without stimulation. The plots for the 'no stimulation' (open red squares) and 'APV' (open blue triangles) cases show an average decrease in spine number (approximately 0.25 spines/100 µm/hour), which is very similar to the baseline spine loss that is described in Figure 2D. The data for the LFS show a

markedly greater spine loss within three hours after the stimulation (2.52 ± 0.43; n = 8), which is significantly different from the control case without stimulation (0.9 ± 0.23; n = 8; p = 0.0074) and APV application with stimulation (0.51 ± 0.29; n = 5; p = 0.0046) over the same time window. Whereas these data quite clearly show that LFS-induced spine retraction is dependent on NMDA receptor activation, they also imply that the baseline spine retractions are independent of NMDA receptor activation.

To be able to characterize in greater detail the kinetics of individual spine retraction events, we repeated the LFS experiments at higher image acquisitions rates (one stack every 10 min as opposed to 30 min). Figure 3D and E show representative examples of timeseries of spine retractions, illustrating that the time it takes for individual spines to retract may range from under 10 min (Figure 3D, upper arrow) to about an hour (Figure 3D, lower arrow).

Low-frequency stimulation does not induce spine growth

Similar to the reasoning above and in light of the fact that TBS is effective at inducing spine growth, we wanted to find out whether a similar 'positive' morphological plasticity is inducible by the LFS protocol. Therefore, we also counted and compared the number of newly grown spines after LFS with the cases of no stimulation and APV application during stimulation. As Figure 3C shows, this protocol did not induce spine growth significantly compared to unstimulated (p = 0.39, two-tailed t-test) or APV-treated (p = 0.62, two-tailed t-test) conditions. This shows that, opposite to the TBS protocol, the LFS protocol is effective at inducing spine retractions, but ineffective at inducing spine growth on dendrites of CA1 pyramidal neurons. The rate of generation of

transient spines (grey symbols and curves) was again indistinguishable between LFS and unstimulated control conditions.

Activity-dependent spine retraction depends on developmental age

We wanted to explore the possibility whether the activity-dependent morphological plasticity described here is prone to developmental changes. It has been proposed that the maturation of slice cultures continues in vitro and that their age roughly corresponds to the age at which the slice culture was prepared plus the days the tissue has been in culture (De Simoni et al., 2003). We therefore repeated the LFS experiments using older slices cultures that had been cultured for varying times ranging from 28 to 54 days in vitro (DIV). We compared the numbers of spine retractions after LFS between the older cultures with those from the original experiments, (DIV 9-18). The effect of the stimulus on spine retractions is quantified in Figure 4A, showing that the number of spine retractions per 100 µm of dendrite in the older slice cultures is reduced by about 65 % compared to the younger ones (0.89 ± 0.26, n = 8 versus 2.52 ± 0.43, n = 8, t240-t60, p = 0.0078, two-tailed t-test). We also quantified the loss of spines under unstimulated conditions in the older slice cultures to be able to assess the degree of baseline loss as a function of the age of the slice culture. Similar to the LFS experiments, the baseline spine loss is markedly reduced by about 65 % in the older slice cultures compared to younger ones (0.31 ± 0.13, n = 5 versus 0.90 ± 0.23, n = 8, p = 0.049), indicating that spines are generally more stable in older cultures. Figure 4B shows the entire set of experiments using the LFS protocol, displaying the number of spines lost three hours after the stimulation for each experiment against the respective age of the slice culture. The straight line is a linear regression through the data, revealing a significant correlation (r =

0.69, $p = 0.0037$). This indicates that the ability of neurons to retract spines in an activity-dependent way indeed decreases with age.

Discussion

We have demonstrated that dendritic spines of hippocampal CA1 pyramidal neurons in organotypic slice cultures exhibit bidirectional activity-dependent morphological plasticity. Using time-lapse TPLSM of GFP-positive neurons, we imaged the dendritic structure before and after extracellular electrical stimulation and assessed its plasticity by counting the numbers of spines that grew or retracted. Our data show that spines can grow de novo or disappear completely depending on the stimulation protocol. Whereas a stimulus that is classically used to induce LTP at the Schaffer collateral synapses (Larson et al., 1986) leads to spine formation, we now find that LFS normally used to induce LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992) induces spine retractions.

The earlier studies on spine formation were careful to state that spinogenesis can not necessarily be equated with synaptogenesis. Even if the formation of spines leads to new synapses, it is not clear whether these spines actually make contact with the correct presynaptic fibers to support the synaptic enhancement that generated them. The case is similar for spine retraction, where it is also possible (1) that the retracted spines do not eliminate their synapses but that a synaptic connection is maintained on the dendritic shaft, (2) that even if synapses are eliminated, it is not clear whether they were part of the connections that underwent LTD. It is therefore an open question whether the activity-dependent loss of spines provides a structural basis for LTD. Since LTD is usually expressed with little delay after LFS, the fact that stimulus-induced spine retractions still occur well after the stimulus may instead point to a homeostatic mechanism that could allow neurons to adjust the synaptic strengths of the remaining synapses to optimal levels

within their dynamic range. At this point it remains to be seen whether the functional down-regulation of synaptic strength can be attributed – entirely or in part – to the observed loss of spines and potentially synapses. Still, the fact that APV can block LFS-induced LTD at Schaffer collateral/CA1 synapses (Dudek and Bear, 1992; Mulkey and Malenka, 1992), as well as LFS-induced spine loss, suggests that both phenomena are closely related.

A further notable parallel between LFS-induced spine loss and LTD is their age-dependent decline. For LFS-induced LTD it has been reported that the largest effects occurs at P12-20, the ability to undergo LTD is reduced considerably at P 31-40, and it completely vanishes from P41 on (Kemp et al., 2000). This time-course beautifully parallels our data (Figure 4B) where we find the strongest spine loss during the first two to three weeks and spine loss progressively vanishes thereafter.

Interestingly, in young cultures we observed a loss of spines in the absence of any stimulation that clearly outweighs the baseline rate of spine formations, suggesting that the overall spine density should decrease continuously. This could reflect a general rundown of the slice culture under our experimental conditions, although we find this unlikely, since single spines are retracted while others remain stable for many hours. Moreover, this baseline loss is much less pronounced in older slice cultures, suggesting a developmental change in the stability of spines and their ability to undergo activity-dependent retraction.

The spine loss might also be explained in the context of a hypothesis originally put forward with respect to synaptic strengthening and weakening (Cooper, 1973) rather than with spine generation and spine loss. Evidently, the creation of new spines (or

stronger synapses) has to be counteracted by some homeostatic mechanism to prevent saturation. One way to achieve this is to have a general unspecific loss of spines (or synapse strength; Cooper, 1973; Palm, 1982) to ensure that the average number of spines (or average synapse strength) is maintained. Whether and how such a mechanism is regulated is an interesting question of its own, however beyond the scope of the present paper. It is thus conceivable that the observed baseline spine loss, subserves such a homeostatic purpose.

Our study confirms and extends previous work by demonstrating that a LTP-inducing stimulus is accompanied by the growth of new spines on dendrites of CA1 pyramidal neurons. In a departure from the study of Maletic-Savatic et al (1999) who reported the outgrowth of filopodial processes after prolonged high-frequency stimulation, we found that TBS led to the growth of dendritic structures resembling bona fide spines. We only very rarely observed the appearance of new filopodia. This is in line with a report from our own laboratory which showed that 'pairing' of pre- and postsynaptic activity under a local superfusion paradigm causes the growth of new spines after the induction of synaptic plasticity (Engert and Bonhoeffer, 1999). However, the number of new spines after LTP-inducing stimulation reported in this earlier study is higher than what we report here. A potential explanation for this discrepancy may be provided by the different stimulation paradigms used in the two studies. Not only is the local superfusion approach (Engert and Bonhoeffer, 1999), by which all non-superfused parts of the dendrite are silenced, quite different from local stimulation which leaves the whole dendrite in its normal state of activity. Also the difference between TBS (used in

the present study) and 'pairing' (used in the previous study) might well account for at least part of the numerical difference in newly generated spines.

Moreover, the spine growth we observe here and its magnitude agrees well with a recent study by Jourdain et al (2003) who report the emergence of approximately one new spine per 100 µm length of dendrite under comparable stimulation conditions. Taken together, these findings lend strong support to the hypothesis that functional synaptic plasticity should be reflected in alterations of neuronal morphology (Cajal, 1911; Hebb, 1949), and open the prospect of studying activity-dependent synapse turnover in real-time in an in vitro system. Whether the synaptic activation-induced growth of new spines can be equated with the formation of new functional synapses has not been determined yet, but other studies (Trachtenberg, 2002) have shown that newly generated spines – albeit not by an activity-dependent paradigm – entail the formation of ultrastructurally mature synapses.

Our study shows that spines can not only be formed de novo but that they can also be retracted in an activity-dependent way. This suggests that the functional plasticity of LTP and LTD is mirrored by bidirectional morphological plasticity. It is tempting to speculate that these morphological changes might be used to stabilize functional changes to make them more permanent. It should be kept in mind, however, that there are other potential functions for these morphological changes and further experiments will be needed to determine their role. The fact that both 'positive' and 'negative' morphological changes occur in concert with their functional counterpart suggest that they have an important role in shaping neural connections.

Methods

Organotypic Hippocampal Slice Cultures and Recording Solutions

Hippocampal slices (300 µm thick) from postnatal day 5-7 transgenic mice (Thy-1 promoter, GFP-M mouse line, courtesy of J. Sanes, Washington Univ., St. Louis) were prepared, embedded in a plasma clot on glass coverslips, and incubated up to 8 weeks in a roller incubator at 35°C, according to the Gähwiler method (Gähwiler, 1981). The age of the slice cultures for the experiments is expressed in days in vitro (DIV) after the preparation and indicated in the text. For the experiments, cultures were transferred into a recording chamber, where they were continuously perfused with carbogenated (95 % O2, 5 % CO¬2) ACSF containing (in mM): NaCl 126, KCl 2.5, $CaCl_2$ 2.8, $MgCl_2$ 0.5, glucose 10, NaH_2PO_4 1.25, $NaHCO_3$ 26, glycine 0.05, pyruvate 1. The temperature was maintained at 35º C and the pH was 7.4. In some control experiments, 50 µM APV was bath-applied for 45 min (30 minutes before and throughout LFS).

Electrophysiology

Patch pipettes were used for electrical stimulation. They were filled with 3 M NaCl and 10 mM of the fluorescent dye calcein immobilized in agar. A chlorided silver wire was used to pass brief current pulses (0.2 ms) of 15-30 µA from a stimulus isolator (WPI, Berlin, Germany) through the patch pipette. The stimulus protocols used for LTP or LTD were: LTP: Theta-burst stimulation (TBS), consisting of 5 trains (200 ms inter-train interval) of 6 pulses delivered at 100 Hz, repeated 5 times every 10 sec; LTD: Low-frequency stimulation (LFS), consisting of 900 pulses delivered at 1 Hz. The tip of the electrode was positioned in the vicinity of the stretch of apical dendrite that was selected

for time-lapse imaging. The minimal distance between the tip of the electrode and the dendrite was kept between 10-20 μm. In separate experiments, we verified that this arrangement of stimulus electrode and stimulation strength reliably produced subthreshold synaptic potentials which were not contaminated by direct stimulation of voltage-gated conductances in the postsynaptic cell.

Microscopy

Time-lapse two-photon-laser-scanning microscopy (TPLSM) was used to image over time the dendritic morphology of GFP-positive CA1 pyramidal neurons. The red excitation light (λ= 840 nm) from a 5 W Mira-Verdi laser system (Coherent, Santa Clara, CA) was routed through a Fluoview 200 scanner (Olympus, Hamburg, Germany), a suitable dichroic mirror (LOT Oriel, Darmstadt, Germany) and a 40× 1.2 NA water immersion objective (Zeiss, Oberkochen, Germany) mounted on an inverted IX70 microscope (Olympus). The power of the excitation light could be adjusted continuously by an acousto-optical modulator (Polytec, Waldbronn, Germany) and its average value at the objective was set to 10-20 mW. The fluorescence was detected by an external photomultiplier tube (R6357, Hamamatsu, Herrsching, Germany). Image acquisition and online analysis was performed by the Fluoview software (Olympus) and image stacks were saved to disk for off-line analysis. The nominal image resolution was 115 nm/pixel in x-y. A piezo-electrical actuator (Physik Instrumente, Karlsruhe, Germany) was used to move the objective in the z axis (Δz = 0.4 μm). Images were Kalman-averaged over three frames. One stack was acquired every 30 min with three stacks recorded before the stimulation and ten stacks afterwards. For the high time resolution experiments, different acquisition rates were used, the highest being one image stack per three minutes. A

reference image was taken at the start of the experiment to facilitate alignment and to keep sample drift over the six hours of the experiment at a minimum.

Image Analysis

4D (x,y,z,t) image stacks were processed and analyzed using the Imaris 4 software (Bitplane, Zürich, Switzerland). Individual stacks were spatially filtered by an edge-preserving algorithm, re-scaled, and baseline-subtracted. To facilitate overview and for display in the figures, the 3D stacks were volume-rendered as 2D images using the blending projection view in Imaris. All image analysis, however, was done by visual inspection of the individual image sections as well as the rendered 2D projections of the image stacks.

A total of 16,201 μm of dendrite was analyzed (395 ± 220 μm per experiment; mean ± SD). Data obtained from total of 41 slices cultures were included in the analysis. Changes in dendritic morphology were divided into three groups: 1. New spines, which were absent in the first image stack(s), emerged at a later timepoint and remained present throughout the rest of the experiment. 2. Transient spines which were not present in the first image stack(s), appeared later and then disappeared again without re-appearing for the duration of the experiment. Typically, transient spines were observed during a single timepoint. In a few instances, they persisted over multiple timepoints. The timepoint of their emergence is plotted in the graphs. Note that the fact that transient spines are usually only seen in only one timepoint means that we must be underestimating the true number of transient spines. 3. Retracted spines, which were present in the first image stack(s), disappeared at a later timepoint, and did not reappear for the duration of the experiment. These three categories were sufficient to fully describe the ‘behavior’ of the spines that

we observed under these experimental conditions. No attempt was made to quantify possible shape or size changes of spines. Rather, we focused on those changes that were all-or-none and that were well within the resolution of TPLSM. To confirm the spine count statistics, 10 out of the 43 experiments were selected at random and subjected to a recount by an operator blind to the experimental conditions. This analysis yielded qualitatively the same results.

Figures

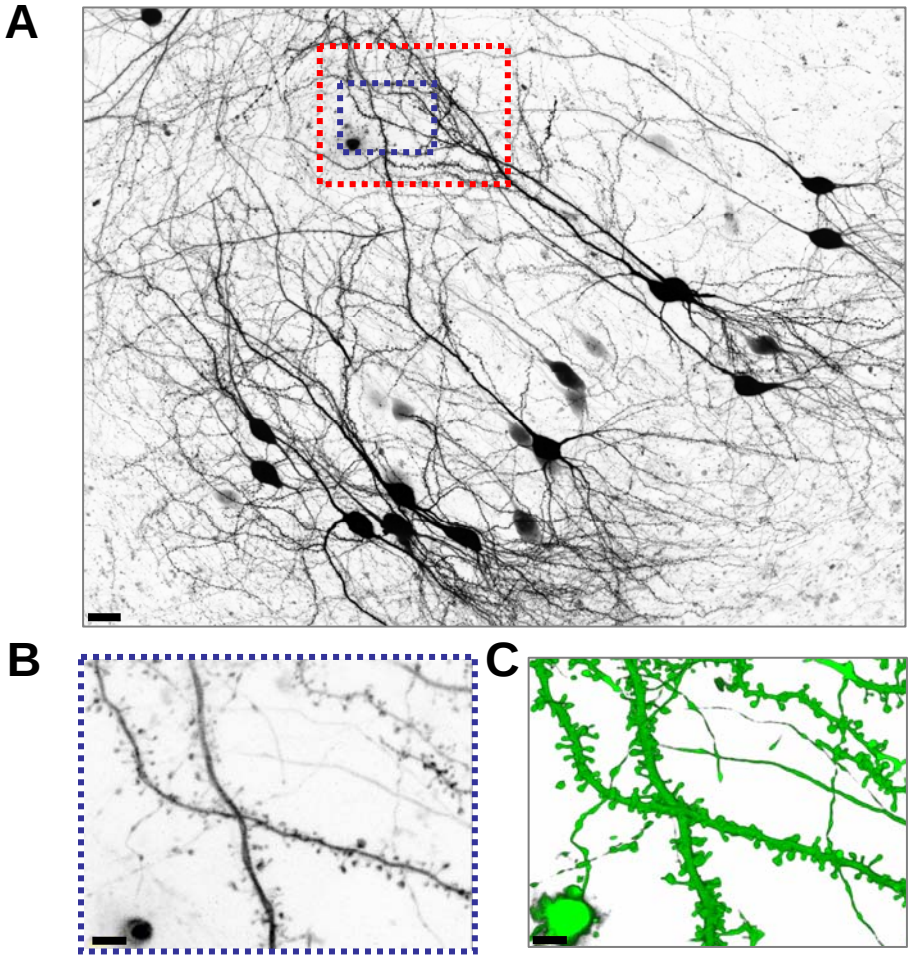

Fig. 1: Nägerl et al (2004)

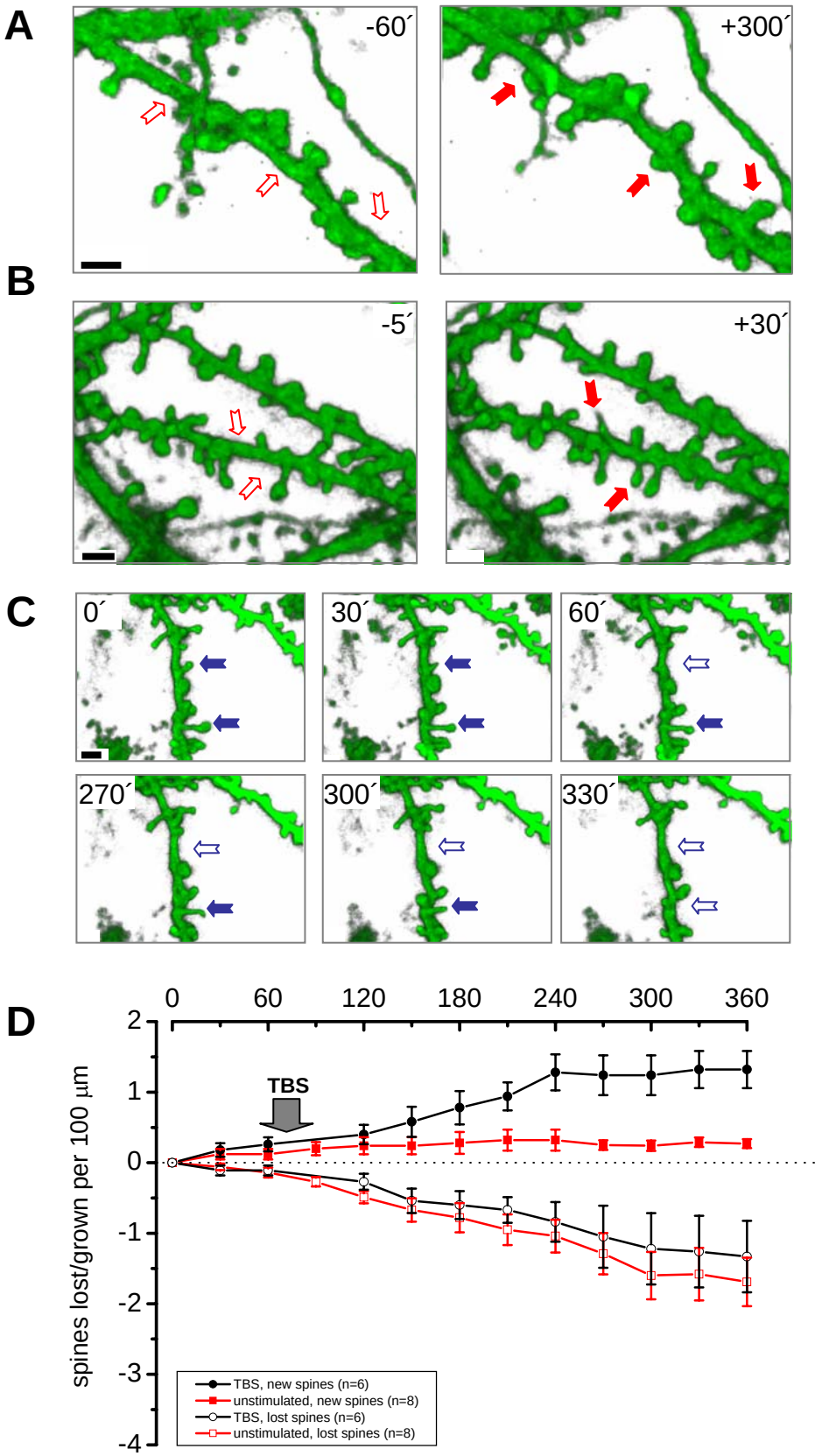

Fig. 2: Nägerl et al (2004)

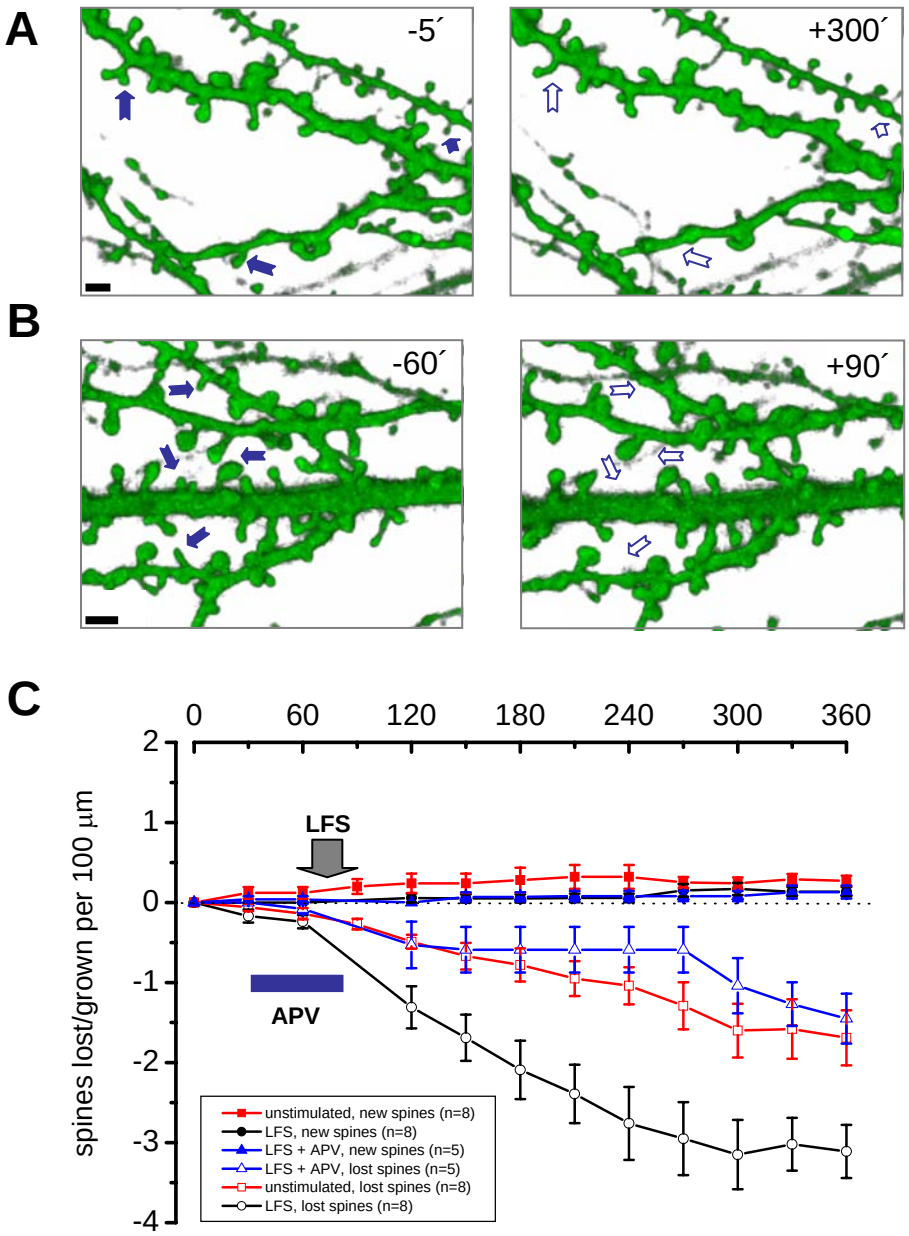

Fig. 3: Nägerl et al (2004)

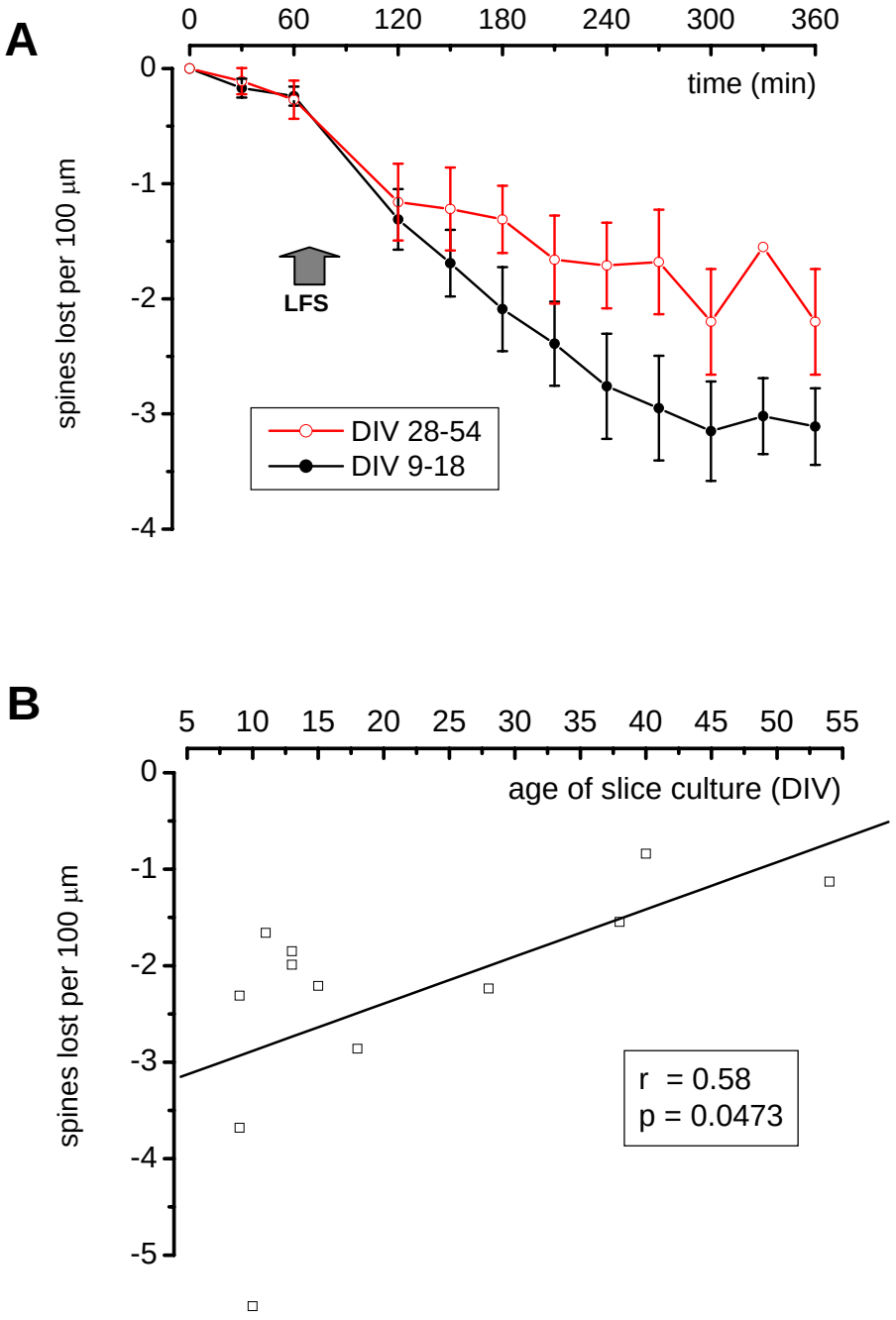

Fig. 4: Nägerl et al (2004)

Figure Legends

Figure 1: TPLSM allows faithful time-lapse imaging of dendritic fine structure.

A Overview of GFP-positive CA1 pyramidal neurons, the red rectangle outlining a typical field of view containing stretches of dendrites and the fluorescent tip of the electrode (green circle) used for extracellular stimulation. Scale bar is 20 µm. Zooming in onto a stretch of dendrite (blue rectangle) either as a raw image (**B**) or after image processing (**C**), illustrates the improvement in image quality achieved by Kalman averaging, filtering and volume-rendering. Scale bars in **B** and **C** are 5 µm

Figure 2: TBS stimulation induces new spine formation.

A Stretch of dendrite shown before (t0 = -60 min) and after (t1 = +300 min) TBS stimulation. The open red arrows in the left panel point at sites of later spine growth, indicated by the solid red arrows in the right panel. **B** Another example of TBS-induced spinogenesis (t0 = 5 min; t1 = +30 min). **C** Example of spontaneous (unstimulated) loss of spines, the solid blue arrows pointing at sites of later spine loss as indicated by the open blue arrows in panels showing images taken at later timepoints. All scale bars are 2 µm. **D** Quantification: Number of spines generated or retracted per 100 µm of dendrite for each timepoint, showing a clear effect of TBS (onset of stimulation indicated by arrow) on the generation of new spines (solid black circles, n = 6) as compared to unstimulated controls (solid red squares, n = 8). In contrast, the number of retracted spines after TBS (open black circles, n = 6) is similar to that of unstimulated controls (open red squares, n = 8). Note, that after TBS, the numbers of new and retracted spines are comparable, indicating that there is no net gain in spine number even after TBS. Also

indicated in the plots are the timepoints of the emergence of transient spines after TBS (open grey circles) and during unstimulated conditions (open grey squares). Error bars indicate SEM.

Figure 3: LFS stimulation induces spine retraction.

A, **B** Representative stretches of dendrites before (-min) and after (+min) LFS. The solid blue arrows on the left panels point at sites of later spine retraction as shown by the open arrows in the right panels. Note that the loss is specific to some spines, sparing other, neighboring spines. All scale bars are 2 µm. **C** Quantification: Numbers of new or retracted spines per 100 µm of dendrite, showing that LFS (onset of stimulus indicated by arrow) leads to a marked decrease in the number of spines (open black circles, n = 8) as compared with unstimulated (open red squares, n = 8) or APV-treated controls (open blue triangles, n = 5, duration of APV application indicated by blue bar). Note that the number of retracted spines is comparable between the unstimulated and APV-treated conditions, indicating that the LFS effect on spine retraction requires NMDA-receptor activation and suggesting that baseline spine loss is NMDA-receptor independent. LFS does not lead to the formation of new spines as indicated by the flat curve displaying the number of new spines/100 µm (solid black circles, n = 8), which is indistinguishable from the curves for the unstimulated and APV-treated control conditions. Also plotted is the time-course of the emergence of transient spines after LFS (open grey circles) and during unstimulated conditions (open grey squares). Error bars indicate SEM. **D**,**E** Representative examples of timeseries of spines in the process of retracting, with the duration ranging from under 10 min to about one hour. The blue-white arrows indicate spines that are in the process of retracting.

Figure 4: LFS-induced spine retraction and baseline spine loss depends on developmental age.

A CA1 pyramidal neurons from older slices cultures (DIV 28-54) exhibit a reduced ability to retract spines after LFS (solid grey symbols; time of stimulation indicated by arrow) compared to younger slice cultures (DIV 9-18; solid black symbols). Note that the loss of spines is much less pronounced in the older slice cultures under unstimulated control conditions, indicating that both, baseline and LFS-induced spine retractions are significantly more frequent in younger slice cultures. **B** Scatter plot of all LFS experiments, plotting the number of spines retracted two hours after LFS (open black squares) against the age of the slice culture. The black line plots the linear regression through the data points, revealing a significant correlation ($r = 0.69$, $p = 0.0037$) between the age and number of spine retractions. Error bars indicate SEM.

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Discussion

Immunohistochemical characterization of activity-dependent spinogenesis in hippocampal neurons

The first part of my dissertation aimed to test whether activity-dependent spine growth leads to the formation of functional synapses. Various studies support the century old proposal that spines, as they carry the postsynaptic specialization of most of the excitatory synapses, constitute the site of learning and long-term stable memory in central neurons (Ramon y Cajal, 1891, 1893). The introduction of novel, high-resolution imaging methods led to several experimental studies demonstrating morphological plasticity of spines under various conditions. A few studies could show that the induction of LTP in a vitro model induces changes in spine morphology and more importantly leads to the formation of new dendritic spines (Nägerl, Eberhorn et al., 2004; Engert and Bonhoeffer, 1999; Toni et al., 1999) or filopodial processes (Maletic-Savatic et al., 1999). However, it is still unclear if and when the new spines or filopodia contain or acquire functional synapses.

Using time-lapse two-photon and confocal microscopy to image GFP-positive pyramidal neurons, I confirmed previous results demonstrating spine growth in an activity-dependent manner. The stimulation paradigm was either local high-frequency stimulation (HFS) in the vicinity of the imaged stretch of dendrite or bath application of a "chemical LTP" induction protocol, which is known to lead to changes in spine length and orientation as well as spine density (Hosokawa et al., 1995). However, LTP was not confirmed electrophysiologically in these experiments. Independent of the type of stimulation, I observed mostly the growth of bona fide spines, while the formation of

filopodial protrusions was seldomly detected although they are often discussed to be the precursors of spines. The average amount of new spines per dendritic length was not measured quantitatively but was on estimation in good accordance with spine values described in the literature (Engert and Bonhoeffer, 1999).

As the model system of choice for investigating activity-dependent morphological changes, I used organotypic slice cultures of the hippocampus of Thy1-GFP mice. Unlike in previous studies, in which the cells had to be impaled and dye-loaded for visualization (e.g. Engert and Bonhoeffer, 1999) the CA1 pyramidal neurons of this transgenic mouse line express endogenous GFP in a subset of neurons which allows tracking the dendritic morphology of individual cells over time. The advantage lies therefore in the non-invasive visualization and in the reliable staining of all dendritic protrusions.

Organotypic cultures combine the advantage of a relatively well preserved connectivity of the neuronal network similar to the intact brain and a high optical "accessibility" which is much better than with acute slices. The cell density of organotypic slice cultures is reduced to a few cells in height and the dendritic trees of the neurons are more or less two-dimensional, advantages which facilitate imaging experiments because much of the dendritic tree can be imaged within a few optical sections.

Despite all of the studies demonstrating alterations in the spine density after LTP-inducing stimulation, there is yet no direct proof that activity-dependent spine growth leads to the formation of functional synapses on these new spines. There are several possible approaches using time-lapse imaging studies of individual spines and synaptic contacts that would help to answer this open question. One possibility is to label active

presynaptic terminals with styryl FM dyes proving the presence of a functional synapse on a new spine. Despite successful results in dissociated cell cultures where novel spines grown upon conditioning stimulation contacting presynaptic terminals have been reported by this method (Goldin et al., 2001) dye staining is not valuable for slice cultures. These cultures contain a three-dimensional meshwork of hundreds cells with millions of synaptic contacts, which makes it hard to discriminate between individual puncta and the high amount of background staining.

Another approach would be to image “pairs” of cells simultaneously and thereby the future pre- and postsynaptic sites of a synaptic contact. Possible for single cell cultures this technique again is hard to use in slice cultures where hundreds of neurons are candidates for building a synapse and one cannot be sure that the appropriate pre- and postsynaptic sites are indeed labeled. The same uncertainty is given during combined time-lapse imaging of the dendritic structure of GFP positive cells and of neurons expressing fluorescently labeled presynaptic molecules. Only if the transfection efficacy of these transgenes would be 100 %, which per se is highly unlikely, all the future presynaptic sites could be observed.

To overcome these difficulties and to be able to answer the question of the presynaptic innervation and molecular composition of individual spines, I established a postimaging high-resolution immunohistochemical staining procedure. Single newly grown spines of GFP positive neurons which were tracked over time prior to fixation were stained for the presynaptic marker synapsin or the postsynaptic marker GluR2, an AMPA receptor subunit which is commonly present in functional and conducting synapses. In dissociated cultured neurons, the use of antibodies usually produces good

staining results, which can be used to decipher a molecular picture of synapse assembly. In three dimensional slice cultures which are thicker compared to dissociated cell cultures and appear denser compared to sectioned brain slices, the standard antibody staining protocol could not be applied and had to be modified for increased penetration. Good results were obtained by incubating the cultures with the primary antibody for an extended time up to 10 days. Using high-resolution confocal imaging of the stained organotypic slice cultures, individual immunohistochemical markers as well as the presynaptic contact of a previously imaged spine could be determined.

Immunohistochemical stainings against the presynaptic marker synapsin in hippocampal slice cultures result in a clear colocalization of mature CA1 spines with presynaptic terminals in 84 % of all mature spines. This value corresponds nicely to what was described in the literature, data showed that 88 % of dendritic spines of CA1 pyramidal neurons in organotypic slice cultures were colocalized with presynaptic terminals (Richards et al., 2005). Only 5 % of all mature spines were clearly not apposed to any presynaptic punctum. The remaining 11 % are “uncertain colocalizations” where the fluorescent signal of both the spine and the immunohistochemical stain was close but did not abut.

Staining mature spines in slice cultures against the postsynaptic marker and subunit of the AMPA receptor GluR2 resulted in a colocalization percentage of 44 %. 33 % of all mature spines did not contain any discernable amount of GluR2. Possibly a higher percentage of spines in organotypic slice cultures do not bare a functional synapse even if the majority of mature spines abut a presynaptic terminal (see above). At least for dissociated hippocampal neurons this seems unlikely since nearly all AMPA receptor

clusters (here identified by the content of subunit GluR1) were associated with synaptophysin (Mi et al., 2002).

In our approach, the lower percentage of colocalization between mature spines and GluR2 signal and the higher percentage of uncertain colocalization (23 %) could also be due to technical reasons. Since the GluR2 staining was not as complete and not as crisp as the synapsin staining, in which puncta were more defined and less fuzzy (compare Figure 2) it was difficult to determine true colocalization according to our stringent criterion for colocalization (see above). Maybe the amount of postsynaptic GluR2 was not enough for gaining this distinct and unambiguous staining signal. In addition, it is possible that the GluR2 immunostaining does not recognize all endogenous GluR2 protein; even if a similar immunohistochemical staining procedure could unambiguously stain for presynaptic synapsin, this protocol may need some further modifications for the reliable detection of GluR2.

The aim of this study was to determine if and when newly grown spines contain fully functional synapses. Synaptic plasticity, induced by either electrical or chemical LTP, leads to de novo spine growth in organotypic slice cultures. Postimaging immunohistochemistry against the presynaptic marker synapsin showed that spines, which emerged upon LTP induction initially do not colocalize with presynaptic synapsin. Therefore, it is very likely that these new spines emerge de novo, not from a preexisting shaft synapse but rather from a new position along the dendrite. The likelihood for a newly induced spine to colocalize with a synapsin punctum increases with the age of the spine. After two hours of existence most new spines show colocalization with synapsin.

Interpreting these data alone one would postulate that these spines colocalizing with presynaptic synapsin finally became part of a synaptic contact. Unpublished post-imaging electron microscopic studies showed that spines newly grown after electrical HFS do not form real synapses within the first 8 hours of existence. However, the newly grown spines did not form synapses themselves but were rather in contact with presynaptic boutons, which formed synapses with other spines in the vicinity (Nägerl et al., 2005, in preparation). Therefore the colocalization of the fluorescent signal of new spines older than two hours with presynaptic synapsin could be due to the close proximity of a new spine and an existing synaptic contact. Possibly, the new spines emerged actively towards pre-existing synaptic contacts, formed by mature spines and axon terminals in the vicinity. It is likely that this mature and innervated spine belongs to a different (and therefore unlabelled) neuron. This assumption goes in line with ultrastructural observations showing that when more than one spine forms synapses with the same presynaptic bouton, they are usually from different neurons (Harris and Stevens, 1989). It is possible that the presynaptic terminal may divide later and forms two separate synaptic contacts, one with the previously existing spine and one with the newly grown spine.

Most of the spines on pyramidal cells contain a postsynaptic density (Moser et al., 1994; Gray, 1959). Furthermore, the majority of mature spines (88 %) in hippocampal slice cultures are part of a synaptic contact (Richards et al., 2005) and also the immunohistochemical staining of the present study shows a colocalization between mature spines and presynaptic synapsin in 84 % of the cases (see above). It is therefore likely that most spines will ultimately form synaptic contacts. The question remains when

after emergence a new spine acquires a synapse. To answer this question newly emerged spines were tested for their content of postsynaptic GluR2, a subunit of the AMPA receptor and therefore a marker for a fully functional synapse. Within the first hours of existence most of these spines do not contain postsynaptic GluR2. These results strengthen the theory that newly grown spines do not contain functional synapses and do not participate in synaptic transmission at least within the first six hours of existence. Future experiments have to follow the colocalization rate between new spines and GluR2 over a longer time of existence.

It is possible that new spines do not lack the complete postsynaptic specialization but go through a transient state of silent synapses before these silent synapses convert in functional and conducting synapses. It has been shown that a significant fraction of glutamatergic synapses are postsynaptically silent and contain NMDA receptors without detectable AMPA receptors (Liao et al., 1999). Such silent synapses can be activated by the recruitment of AMPA receptors after titanic stimulation (Liao et al., 1995), thereby altering the overall synaptic strength of the cell. Perhaps the lack of immunopositive staining of GluR2 and thereby AMPA receptors in the newly grown spines marks their identity as parts of silent synapses. Furthermore, it is thinkable that LTP induction leads to the emergence of spines at sites of previous silent shaft synapses along the dendrite. To follow that possibility additional immunohistochemical stainings against the NMDA receptor (i.e. the NR1 subunit) in combination with synapsin and GluR2 immunostainings are necessary.

In summary, this study suggests that spines grown upon the induction of LTP in the hippocampus emerge de novo and not from an existing shaft synapse. Most of the

new spines did not contain functional synapses within the first six hours of existence while the colocalization with presynaptic terminals increases with the age of the spine, resulting in a colocalization rate of 84 % in mature spines. It is therefore thinkable that the new spines protrude towards an existing synaptic contact, possibly forming functional connections later.

Bidirectional Activity-Dependent Morphological Plasticity in Hippocampal Neurons

The second part of my dissertation was to investigate whether synapse depression by the induction of LTD, the possible in vitro correlate of memory erasure, also induces morphological spine modifications. Electrophysiologically, bidirectional effects are well known, namely long-term potentiation (LTP) after high-frequency stimulation (HFS) and long-term depression (LTD) after low-frequency stimulation (LFS) (Bliss and Lomo, 1973; Dudek and Bear, 1992; Mulkey and Malenka, 1992; Liu et al., 2004). Consequently, one may expect that LTD-inducing stimulation should be correlated with spine pruning or at least reduction in size if LTP-inducing stimuli in reverse lead to spine growth.

Experiments were done to investigate this possibility by imaging the morphology of dendritic spines after LTD-inducing stimulation over time (in collaboration with Dr. U.V. Nägerl). To this end, I did time-lapse imaging of dendritic spines of green fluorescent protein (GFP) positive CA1 pyramidal neurons with two-photon laser-scanning microscopy (TPLSM). The fate of spines before and after LFS, which is classically used to induce LTD at the Schaffer collateral/CA1 synapses, was monitored for up to six hours. The results clearly demonstrated that LFS indeed induces spine retractions, which was the first experimental study to show this effect.

In addition, previous data were confirmed, demonstrating that a LTP-inducing stimulus is accompanied by the growth of new spines on dendrites of CA1 pyramidal neurons (Nägerl, Eberhorn et al., 2004; Engert and Bonhoeffer, 1999). Maletic-Savatic et al. (1999) reported enhanced outgrowth of predominantly filopodial processes after local HFS while little spine growth was reported. The extension of these filopodial protrusion

started about 20 min after HFS and the filopodium persisted for at least 40 min; the growth was restricted to the close vicinity of the stimulation electrode and was prevented by APV application. In contrast, Engert and Bonhoeffer (1999) reported in hippocampal slice cultures the outgrowth of mainly spines and the extension of existing spines in CA1 pyramidal neurons 30-60 min after 'pairing' of pre- and postsynaptic activity under a local superfusion paradigm. Our results are in line with the results of Engert and Bonhoeffer, as new filopodia were rarely observed while most of the new protrusions were bona fide spines. However, the average number of new spines after LTP-induction in our experiments was 3 per 100 µm dendrite, which is half of what was described in this previous study but is in line with results from Jourdain et al. (2003) who reported similar numbers of new spines under comparable stimulation conditions. The discrepancy to the superfusion experiment may be due to the different stimulation paradigms used or to the different overall experimental design.

Growth or retraction of dendritic spines is directly related to the LTP- or LTD-inducing stimulation, respectively. In the absence of any stimulation the number of spontaneously retracted spines was higher that the number of newly grown spines, suggesting that the overall spine density decreases continuously. This instance appears to be development-dependent since it was most prominent in younger cultures (during the first 2-3 weeks) while this baseline loss was less pronounced in older cultures (older than three weeks). This general slight rundown in spine density may be partly due to the reduced innervation of the hippocampal neurons in slice preparations, compared to the in vivo situation. Also the ability of a neuron to retract spines after LFS shows an age-dependent decline: the strongest LFS-induced spine loss occurs in young cultures (up to

three weeks in vitro). These data are very well in line with previous studies (Kemp et al., 2000) showing that also LFS-induced LTD has the largest effect at postnatal day 12-20.

Taken together, this study shows that dendritic spines of hippocampal CA1 pyramidal neurons in organotypic slice cultures exhibit not only bidirectional functional synaptic plasticity, but also bidirectional activity-dependent morphological plasticity, depending on the stimulation protocol.

While we observed the emergence and disappearance of entire spines, Zhou et al. (2004) could demonstrate a 25 - 40 % reduction in the diameter of spine heads after LTD-induction, an effect which persisted for one hour after stimulation. On the other hand they reported an increase in the spine head diameter after high-frequency stimulation. This study nicely corroborates the bidirectionality of activity-dependent morphological plasticity of dendritic spines as they showed the symmetry between changes in spine morphology and synaptic responses. However, these data of spine shrinkage following LTD induction were based on small fluorescence changes. In fluorescent microscopy the optical resolution limits the detection of subtle morphological changes such as spine shrinkage or enlargement, and this method is therefore not ideal for measuring this type of effect. However, we could also monitor the process of ongoing activity-dependent spine retraction, which lasted from under 10 minutes to about one hour, but was not quantitatively analyzed.

The fact that these changes in spine morphology occur raises the question about some of the molecular mechanisms behind. Shortly before the present study from our laboratory was published (Nägerl, Eberhorn et al., 2004), Okamoto et al. (2004) demonstrated that the two different forms of synaptic plasticity, LTP and LTD, cause the

enrichment of different forms of actin in the dendritic spines. LTP was associated with an increase in the amount of filamentous (F-) actin while LTD was associated with spine shrinkage and an increase in the amount of globular (G-) actin. Thus, they showed that LTP is associated with an increase in F-actin and thereby repeated previous in vivo data (Fukuzawa, 2003). The authors speculate that the polymerization of actin, which acts as the primary cytosceletal element, together with the subsequent insertion of postsynaptic receptor proteins, causes the enlargement of the spine head. In contrast, the shift to a lower F-actin/G-actin ratio in the spine head in response to LTD is likely to result in the loss of postsynaptic cytosceletal integrity, comprising the proteins anchored to actin. This circumstance might be also responsible for the reduced number of synaptic AMPA receptors in the spine (Carroll et al., 1999; Luthi et al., 1999) after LTD induction, together with a general destabilization of the spine (Passafaro et al. 2003), the shrinkage of its synaptic surface, and the observed complete spine retraction.

One major question remains: Can spinogenesis be equated with synaptogenesis? Trachtenberg et al. (2002) have shown that newly formed spines – albeit not by an activity-dependent paradigm – have the appearance of ultrastructurally mature synapses. But what about spines grown upon synaptic activation, do they contain new functional synapses? Analogous to previous studies (Engert and Bonhoeffer, 1999), I did not verify if the new spines formed upon HFS carry functional synapses. Even if the formation of spines eventually leads to new synapses, it is not clear whether these spines indeed make contact with the correct presynaptic fibers to support the synaptic enhancement that generated them.

The case is similar for the opposite effect, namely spine retraction after LFS. What happens to the (presumable functional) synaptic contact if a spine is pruned upon LTD? It is thinkable that the retracted spines did not contain any functional synaptic contact beforehand. Perhaps the pruning the spine after LTD induction may ultimately remove functionally useless dendritic spines which are not participating in the neuronal circuit.

However, most of the spines on pyramidal neurons in the hippocampus form excitatory synapses (Moser et al., 1994). It is possible that after LTD induction the synaptic connection is still maintained on the dendritic shaft as a shaft synapse, although the spine was retracted. On the other hand, the pre-and postsynaptic specializations could first separate upon LFS and then potentially disappear. Postimaging immunohistochemistry after LTD for a functional synaptic marker could at least answer the question whether spine retraction is correlated with synapse elimination. In pilot studies doing postimaging immunohistochemistry against the GluR2 subunit of the AMPA receptor as a marker of functional synapses I could observe that at the sites of previous spines, retracted after LTD induction, an immunopositiv GluR2 punctum remained at the dendrite. The Figure shows a dendritic stretch before (Figure **A**) and after (Figure **B**) spine retraction. The corresponding dendrite after fixation and immunohistochemistry is shown in a maximum intensity projection in Figure **C**, Figure **D** demonstrates that a GluR2 positive remains at the site of the previous spine. It is possible that the synapse of a retracted spine is maintained as a shaft synapse, or that at least the postsynaptic content of GluR2 remains. These results need to be verified in future experiments and could present one way to solve this open question.

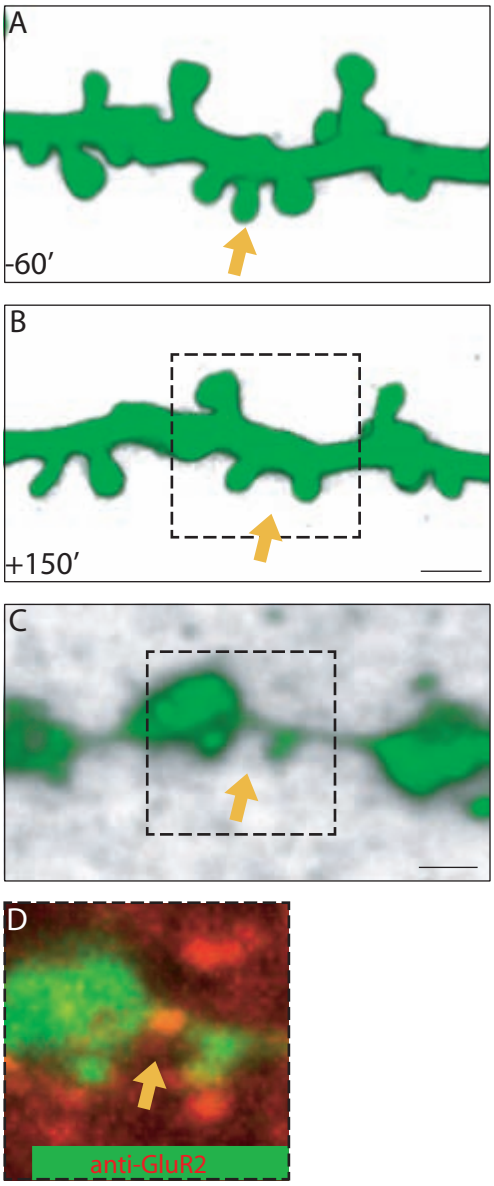
A
-60′
B
+150′
C
D
anti-GluR2

Figure: *GluR2 positive staining at the dendritic site of a retracted dendritic spine.*

A *and* ***B*** *TPLSM images of dendritic stretches before (- min) and after (+ min) LFS. The arrow indicates the retracted spine, pruned at time point + 60 min.* ***C*** *maximum intensity projection of the corresponding dendrite after fixation and immunohistochemistry.* ***D*** *shows that a clear GluR2 positive punctum (in red) remains at the site of the retracted spine. Scale bars, 2 µm.*

Nevertheless, even if the pruned spine loses its afferent innervation – which could possibly be confirmed by postimaging immunohistochemistry- it is not proven that the activity-dependent loss of spines and potentially synapses is the morphological basis of functional down-regulation of synaptic strength upon LTD. Comparing the sequence of events it is obvious, that the stimulus-induced spine retractions occur very well after the stimulus and also with a certain delay after LTD expression. It is therefore unlikely that the spine shrinkage or retraction is the absolute mechanism for LTD. Rather, the stimulus-dependent spine retraction may be a homeostatic mechanism that could allow neurons to adjust the synaptic strength of the remaining synapses to optimal levels within their dynamic range. Furthermore, Zhou et al. (2004) saw that the shrinkage of spines does not depend on the elevation of the downstream effector PP1, which is required for LTD. Clearly the NMDA receptor blocker APV prevents both LFS-induced LTD (at Schaffer collateral/CA1 synapses; Dudek and Bear, 1992; Mulkey and Malenka, 1992) as well as LFS-induced spine loss (Nägerl, Eberhorn et al., 2004; Zhou et al., 2004) demonstrating the close relationship between both phenomena.

Taken together, this study shows for the first time that the functional plasticity of LTP and LTD is mirrored by bidirectional morphological plasticity of spines. This finding supports the decades old hypothesis that functional synaptic plasticity should be reflected in alterations of neuronal morphology (Cajal, 1911; Hebb, 1949). Perhaps these

morphological changes are used to stabilize functional changes making them more permanent and thereby play an important role in the refinement and shaping of neural connections. The elimination of connections, which had undergone LTD could represent a mechanism of how neurons could specifically remove redundant connections.

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Abbreviations

ACSF	Artificial Cerebrospinal Fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APV	2-amino-5-phosphonopentanoic acid
CA1	Cornus Ammonis 1
CA3	Cornus Ammonis 3
DIV	Days in vitro
EGFP	Enhanced Green Fluorescent Protein
EPSP	Excitatory Postsynaptic Potential
GluR2	AMPA receptor subunit 2
HFS	High Frequency Stimulation
LFS	Low Frequency Stimulation
LTD	Long-term Depression
LTP	Long-term Potentiation
NMDA	N-methyl-D-aspartate
NR1	NMDA receptor subunit 1
PFA	Paraformaldehyde
PSD	Postsynaptic Density
TBS	Theta Burst Stimulation
TPLSM	Two-photon Excitation Laser Scanning Microscopy

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Curriculum Vitae

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Publications

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Society for Neuroscience (sfn), 2004, San Diego, USA

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Ehrenwörtliche Versicherung:

Ich versichere hiermit ehrenwörtlich, dass meine Dissertation mit dem Titel

„Functional and Morphological Plasticity of Dendritic Spines in the Hippocampus"

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Der von mir geleistete Eigenanteil an den beiden Einzelartikeln wurde von meinem Betreuer Prof. Bonhoeffer und den übrigen Coauthoren deutlich dargelegt.

Erklärung:

Hiermit erkläre ich, dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

München, den 01.08.2005

Nicola Eberhorn