Analysis of activity-dependent morphological plasticity of dendritic spines on hippocampal neurons

Dissertation

zur Erlangung des Grades eines Doktors

der Naturwissenschaften

der Fakultät für Biologie

der Ludwig-Maximilians-Universität München

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1. Introduction

Nervous systems acquire information via sensory organs, process this information, and generate an output response. They can modify the output response on the basis of previous experience and store this modification over time. This ability is generally referred to as learning and memory.

Learning and memory processes can be observed in all organisms with nervous systems and therefore are a fundamental characteristic of nervous systems. The mechanisms for learning and memory have been subject to intense speculation. Ramón y Cajal has already suggested that little dendritic protrusions, which he called 'espinas' (spines) and which he believed to connect axons and dendrites (Ramón y Cajal, 1891), are involved in learning (Ramón y Cajal, 1893). Subsequently, Donald Hebb postulated that regulating the strength of connections between nerve cells could be the cellular basis for learning and memory (Hebb, 1949).

In line with these theoretical proposals, it has been experimentally confirmed that the transmission efficacy at synaptic connections can be up- or downregulated (synaptic plasticity) and it is now widely acknowledged that synaptic plasticity is involved in mediating learning and memory processes (Morris et al., 1986; Moser et al., 1998). Furthermore, changes of spine number and morphology have been implied in synaptic plasticity (Engert and Bonhoeffer, 1999).

In spite of these findings, the exact role of dendritic spines during synaptic plasticity and, ultimately, during learning and memory is still unclear. Therefore, studying the relationship between morphological changes of dendritic spines and synaptic plasticity is important to understand the cellular mechanisms that underlie learning and memory.

1.1. Synaptic transmission and plasticity in the hippocampus

The hippocampus is generally accepted to be important for different forms of learning and memory (Becker et al., 1980; Morris et al., 1982; Eichenbaum et al., 1986). Anatomically, it is part of the temporal lobe of the cerebral cortex (**Fig. 1A**). It receives input from the entorhinal cortex, the contralateral hippocampus, the hypothalamus, and the basal forebrain. Output fibers project to the entorhinal cortex and the contralateral hippocampus. The hippocampus has only one cell layer (*stratum (str.) pyramidale*) which contains mainly pyramidal neurons (**Fig. 1A**, **B**). On the basis of morphological differences between the pyramidal neurons, the hippocampus can be divided into four different regions (*cornus ammonis*; CA1-4).

Input into the hippocampus is transmitted via the granule cells of the *gyrus dentatus* to pyramidal neurons in CA3. Axons of CA3 neurons (Schaffer collaterals) project to area CA1 where they form synapses with the apical and basal dendrites of pyramidal neurons in the *str. radiatum* and *str. oriens*, respectively. This relatively simple 'trisynaptic pathway' of excitation and its importance for learning and memory processes make the hippocampus well suited to study synaptic transmission and plasticity.

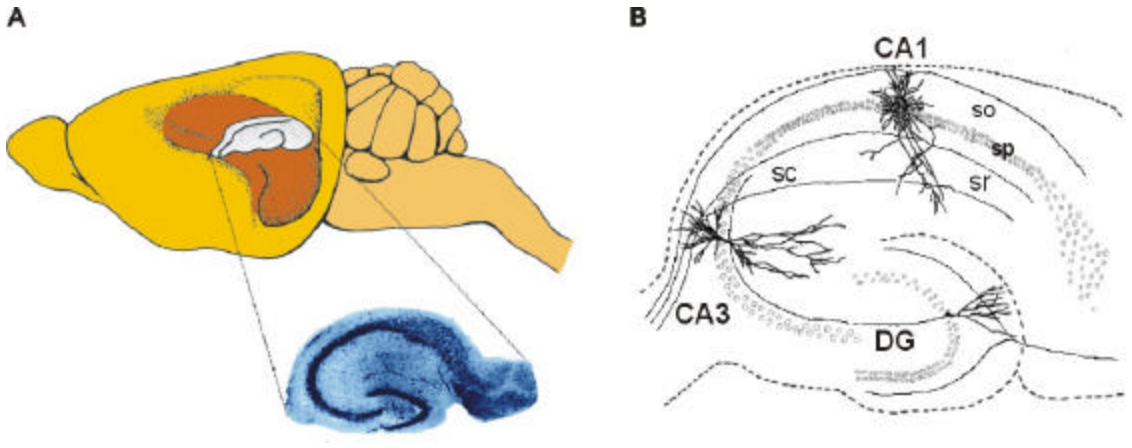

Fig. 1: Position and structure of the hippocampus.
A: Position of the hippocampus (dark red) within the rat brain. Magnified image: Nissl staining of a transversal section (cell layer: dark blue). B: Schematic drawing of a transversal section. DG: Gyrus dentatus, sc: Schaffer collaterals, sr: str. radiatum, so: str. oriens, sp: str. pyramidale.

Synapses are anatomically and functionally specialized structures, where action potentials are transmitted from the axon of one neuron to the dendrite or cell body of another neuron (synaptic transmission). They consist of an axonal (presynaptic) and a dendritic (postsynaptic) specialization which are spatially separated (**Fig. 2**). The postsynaptic site can be located directly on dendrites or on tiny protrusions emerging from the dendrites (spines).

On the presynaptic site action potentials can cause fusion of small membranous vesicles with the presynaptic membrane and release of neurotransmitter from these vesicles (**Fig. 2**). The neurotransmitter molecules diffuse across the synaptic cleft to the postsynaptic membrane where they bind to receptor molecules. The opening of these receptors causes excitatory or inhibitory postsynaptic potentials (EPSPs, IPSPs) via ionic currents into and out of the cell.

In the hippocampus excitatory synaptic transmission relies on the neurotransmitter glutamate and its binding to a particular subtype of postsynaptic glutamate receptors, the a–amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPARs).

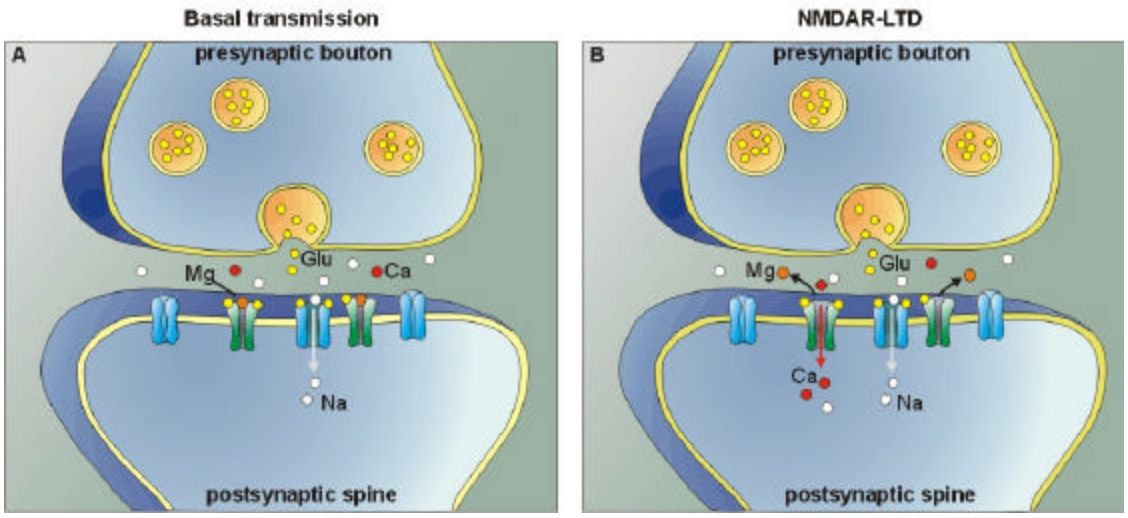

Fig. 2: Schematic view of synaptic transmission at an excitatory synapse.
A, During basal synaptic transmission glutamate (yellow dots; Glu) binding to AMPARs (blue) leads to Na^+ influx (white dots) and depolarization of the postsynaptic spine. NMDARs (green) are inactivated by a Mg^{2+} block (orange dots). **B**, During NMDAR-LTD NMDARs are also activated and mediate influx of Ca^{2+} (red dots).

Ever since the discovery that the magnitude of EPSPs can be potentiated with high-frequency electrical stimulation (Bliss and Lømo, 1973), it has become clear that synaptic transmission can be modulated by neuronal activity. Different stimulation paradigms can induce persistent synaptic potentiation or synaptic depression, termed long-term potentiation (LTP) and long-term depression (LTD).

As the discovery of LTP preceded the one of LTD, LTP has long been considered to be the main cellular mechanism for learning and memory. Accordingly, LTD was believed to be simply the reverse process of LTP. However, the importance of LTD in learning and memory is illustrated by the observation that impaired LTD can also reduce learning and memory performance (Migaud et al., 1998). The relevance of LTD in its own right is further confirmed by the fact that LTD does not simply reverse the activation of signaling pathways of LTP but utilizes distinct signaling pathways on its own.

1.2. LTD

Activity-dependent depression of synaptic transmission was first reported in hippocampal slices when it was demonstrated that the induction of LTP in one pathway led to depression in a second, non-potentiated pathway (heterosynaptic depression) (Lynch et al., 1977). Subsequently, depression in the input pathway (homosynaptic depression) was first described in the form of depotentiation when it was shown that low frequency stimulation (LFS) could reverse LTP (Barrionuevo et al., 1980). Subsequently, LTD in the hippocampus could be induced without prior LTP induction (Dudek and Bear, 1992; Mulkey and Malenka, 1992). Since then, LTD has been reported in slices from various brain regions, including the visual (Artola et al., 1990) and the prefrontal cortex (Hirsch and Crepel, 1991), the amygdala (Wang and Gean, 1999), and the cerebellum (Hansel and Linden, 2000). In addition to brain slices, both LTP and LTD have also been found in the living, intact brain (*in vivo*) (Heynen et al., 1996).

Various different induction protocols have been found to induce LTD, which might reflect the existence of different forms of LTD relying on distinct molecular mechanisms. In the hippocampus, at least two different forms of LTD have been observed: one of them

depends on the activation of N-methyl-D-aspartate- glutamate receptors (NMDARs), the other on the activation of metabotropic glutamate receptors (mGluRs).

1.2.1. NMDAR-LTD

Initially, homosynaptic NMDAR-dependent LTD (NMDAR-LTD) in the hippocampus was demonstrated using LFS consisting of 900 stimuli at a frequency of 1 Hz (Dudek and Bear, 1992; Mulkey and Malenka, 1992). Under this regime, NMDAR-LTD is most easily induced in slices from young animals (Dudek and Bear, 1993). However, in the adult hippocampus application of 900 paired pulses at 1 Hz (Kemp and Bashir, 1997) or trains of stimuli at 5-10 Hz for 15 min (Berretta and Cherubini, 1998) can also induce NMDAR-LTD.

NMDARs are activated by simultaneous membrane depolarization and glutamate binding (Mayer et al., 1984) and are permeable to Ca^{2+} when activated (Dingledine, 1983) (**Fig. 2**). Both NMDAR-LTP (Collingridge and Bliss, 1987; Malenka et al., 1988) and NMDAR-LTD (Mulkey and Malenka, 1992) require an increase in Ca^{2+} concentration ($[Ca^{2+}]$) via NMDARs. This led to the question of how an increase in $[Ca^{2+}]$ within the cell can mediate synaptic potentiation, as well as depression. To explain this phenomenon a model has been developed, which proposes that high levels of $[Ca^{2+}]$ are required for NMDAR-LTP, whereas moderate $[Ca^{2+}]$ levels mediate LTD (Bienenstock et al., 1982; Lisman, 1989). Strong increases in $[Ca^{2+}]$ as a result of high frequency stimulation activate Ca^{2+}-dependent kinases including Ca^{2+}/calmodulin-dependent kinase II (CaMKII). CaMKII can phosphorylate various other molecules, including protein kinase C (PKC) (Routtenberg et al., 1986) and AMPARs (Barria et al., 1997a; Mammen et al., 1997).

In contrast, moderate increases in $[Ca^{2+}]$ are thought to activate phosphatases, including phosphatase 1 (PP1) and calcineurin (PP2A) via the Ca^{2+}/calmodulin complex (Mulkey et al., 1994). In line with this, it has been reported that inhibitors of PP1 and 2 block NMDAR-LTD (Mulkey et al., 1993) and that phosphatase activity increases after NMDAR-LTD induction (Thiels et al., 1998), which leads to CaMKII- and AMPAR-dephosphorylation (Lee et al., 2000).

Phosphorylation and dephosphorylation of AMPARs by protein kinase and phosphatase activity, respectively, changes the receptor properties and regulates synaptic plasticity. Basal synaptic transmission requires phosphorylation of AMPARs at a site which is phosphorylated by cAMP-dependent protein kinase (PKA). NMDAR-LTP is linked to an additional phosphorylation at a CaMKII-dependent phosphorylation site, whereas LTD requires dephosphorylation at both sites (Barria et al., 1997b; Kameyama et al., 1998; Lee et al., 1998). Dephosphorylation at the PKA-dependent phosphorylation site reduces the opening-probability of AMPARs and thus provides a potential mechanism for NMDAR-LTD (Banke et al., 2000). Additionally, dephosphorylation of the CaMKII-dependent site, as it is thought to occur during depotentiation (Lee et al., 2000), has been linked to reduced AMPAR-conductances (Derkach et al., 1999).

In addition to regulation of conductance and opening-probability of existing AMPARs, changes in receptor number have also been implicated in synaptic plasticity. The first indication for this mechanism came from the observation of synapses, at which baseline stimulation evoked only NMDAR-mediated postsynaptic currents but no AMPAR currents ('silent synapses'). Silent synapses were transformed into functional synapses containing AMPAR and NMDAR after NMDAR-LTP (Isaac et al., 1995; Liao et al., 1995). The current view holds that AMPARs are inserted into the membrane during NMDAR-LTP (Shi et al., 1999) and removed during LTD by endocytosis (Kandler et al., 1998; Carroll et al., 1999). In line with this, LTD has been found to depend on the interaction between AMPARs and N-ethylmaleimide-sensitive fusion protein (NSF), a molecule involved in vesicle endocytosis (Luthi et al., 1999).

Another potential mechanism that could underlie NMDAR-LTD is removal of synapses. Indirect evidence comes from studies reporting reduction in spine number or size upon application of NMDA which supposedly caused NMDAR-LTD (Halpain et al., 1998) (Segal, 1995; Lee et al., 1998).

Conversely, NMDAR-LTP has been reported to lead to formation of new spines (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999) and changes in synapse morphology (Toni et al., 1999).

Ultimately, NMDAR-LTD and -LTP manifest themselves via an increase in gene expression. Application of the translation inhibitor anisomycin resulted in an impaired potentiation ~1h after LTP induction, suggesting that there is an early phase of LTP, which is protein synthesis independent, and a late phase of LTP, which requires transcription of mRNA (Frey et al., 1988; Nguyen et al., 1994). With respect to NMDAR-LTD the role of protein synthesis is not yet clear. Using an organotypic culture system, in which hippocampal slices are placed onto a porous filter and maintained in culture for weeks (Müller cultures; Stoppini et al., 1991), it was shown that application of transcription inhibitors impaired NMDAR-LTD (Kauderer and Kandel, 2000). In contrast to NMDAR-LTP, which requires protein synthesis not before 1 h after induction, protein synthesis inhibition in these cultures affected LTD immediately after induction. However, in acute hippocampal slices NMDAR-LTD was found to be protein synthesis-independent (Huber et al., 2000).

1.2.2. mGluR-LTD

Another form of hippocampal LTD depends on metabotropic glutamate receptors (mGluRs) rather than NMDARs (Stanton et al., 1991, Bolshakov and Siegelbaum, 1994). This form of LTD (mGluR-LTD) is readily induced by brief application of mGluR-agonists (Palmer et al., 1997) or by delivering 900 paired stimuli (Kemp and Bashir, 1999). mGluRs are GTP-binding protein (G-protein) coupled receptors mediating Ca^{2+} release from internal stores via different signaling pathways. They come in a variety of subtypes (mGluR1-8) and are classified into three groups (I-III) (Nakanishi et al., 1994). The signaling pathways, which mediate the effects of mGluR activation, have not been documented in detail. It is thought that mGluRs can activate multiple pathways simultaneously as they have been found to bind to several different G-proteins. Activation of mGluR1 has been implicated in inositol 1,4,5-triphosphate (IP_3) and cyclic adenosine-mono-phosphate (cAMP) accumulation, subsequent Ca^{2+} release from internal stores (Aramori and Nakanishi, 1992) and activation of the mitogen-activated protein kinase (MAPK) pathway (Roberson et al., 1999) (**Fig. 2**). Furthermore, mGluR1

activation initiates signaling cascades which involve phospholipase C (PLC), PKC and modulation of NMDAR function (Skeberdis et al., 2001).

Especially mGluR1 and 5, which both belong to group I receptors, have been implicated in mediating synaptic depression in the hippocampus. They are selectively activated by (RS)-3,5-dihydroxyphenylglycine (DHPG) (Schoepp et al., 1994) and are coupled to inositol-phospholipid hydrolysis. In contrast to group II and III receptors, group I receptors are expressed postsynaptically in CA1 neurons (Lujan et al., 1996). Studies using specific antibodies to mGluR1 and mGluR5 have revealed that blocking mGluR1 suppresses DHPG-induced $[Ca^{2+}]$ increase and membrane depolarization of the cell. Blocking mGluR5, however, prevents DHPG-induced suppression of Ca^{2+}-induced K^+ currents and potentiation of NMDAR currents (Mannaioni et al., 2001). Thus, both receptors seem to have distinct roles in regulating neuronal excitability.

Brief application (10 min) of 100 µM DHPG leads to long-lasting (> 1 h) synaptic depression (Palmer et al., 1997). Interestingly, mGluR antagonists can reverse mGluR-LTD even hours after DHPG-washout indicating a continuous role of mGluRs in this form of LTD (Palmer et al., 1997). It has been suggested that mGluR-LTD is mechanistically distinct from NMDAR-LTD as both forms are not mutually occlusive (see Results) and only NMDAR-LTD can reverse NMDAR-LTP (Oliet et al., 1997). Furthermore, the signaling pathways are distinct for both forms of LTD. Both depend on postsynaptic membrane depolarization, a rise in $[Ca^{2+}]$, and activation of PKC but mGluR-LTD does not require phosphatase activity (Oliet et al., 1997). Activation of mGluRs has also been reported to modulate a number of neuronal ion channels, including AMPAR and NMDAR, $GABA_A$ receptors and voltage-sensitive Ca^{2+}- and K^+-channels (for review, s. Conn and Pin, 1997).

Both a presynaptic (Fitzjohn et al., 2001) and a postsynaptic locus (Huber et al., 2000; Snyder et al., 2001; Xiao et al., 2001) of mGluR-LTD expression have been suggested. Presynaptic vesicle release probability was found to be reduced after mGluR-LTD (Zakharenko et al., 2002). On the postsynaptic side, rapid internalization of AMPARs and NMDARs was demonstrated to occur upon DHPG-induced mGluR-LTD (Xiao et al., 2001; Snyder et al., 2001).

In contrast to NMDAR-LTD, mGluR-LTD has been found to depend on protein synthesis in acute slices (Weiler and Greenough, 1993). In the presence of the translation inhibitor anisomycin mGluR-LTD is reduced early after induction (Huber et al., 2000). Therefore, mGluR-LTD requires protein synthesis at or shortly after the time of induction. Protein synthesis seems to be required to regulate glutamate receptor trafficking as the internalization of AMPARs and NMDARs is dependent on protein synthesis (Snyder et al., 2001). It is not clear whether or not mGluR-LTD in organotypic cultures also requires protein synthesis.

Taken together, two forms of LTD exist in the hippocampus utilizing different signaling pathways. However, the relationship between them is not known. NMDAR-LTP has been shown to require activation of mGluRs (Bashir et al., 1993; O'Connor et al., 1994). It is unclear whether or not NMDAR-LTD also depends on mGluR activation.
NMDARs and mGluRs have been found to be expressed at the same synapses: NMDARs are localized at the center and mGluRs at the periphery of the synapse (Baude et al., 1993; Lujan et al., 1997). Thus, it is reasonable to assume that also NMDAR- and mGluR-LTD can occur at the same synapses.
Although they seem to utilize different signaling pathways during induction, NMDAR- and mGluR-LTD might rely on similar mechanisms for LTD expression, as is suggested by the observation that they both can modulate AMPARs and AMPAR trafficking. Thus, it could be speculated that, *in vivo*, NMDAR-LTD and mGluR-LTD do not represent different forms of LTD but rather that both contribute to synaptic depression by two parallel induction pathways.

As mentioned above, structural changes in dendritic morphology have been implicated in mediating NMDAR-LTD and -LTP. Most excitatory synapses in the brain occur on dendritic spines and these structural specializations are known to influence synaptic transmission. Therefore, changes in the number or morphology of dendritic spines could contribute to changes in synaptic efficacy.

1.3. Dendritic spines

Dendritic spines are small protrusions from dendrites that have a single synapse at their tip and account for 90% of excitatory synaptic transmission in the brain (Harris and Kater, 1994). In the hippocampal CA1 area a typical spine usually consists of a narrow neck (diameter: 0.04-0.5 µm) and a more voluminous head. Spine length is in the range of 0.2-2 µm, resulting in a spine volume of 0.004-0.6 μm^3 (Harris and Stevens, 1989).

Spines may serve several functions: First, spines impose a diffusion resistance on molecules such that the narrow spine neck impairs diffusion of molecules into and out of the spine. The compartmentalization of synapses may help to retain molecules at the synapse and prevent their diffusion (Wickens, 1988). In particular, it has been shown that Ca^{2+}-influx upon synaptic stimulation is restricted to single spines without affecting neighboring synapses (Majewska et al., 2000) (for review, see Sabatini et al., 2001). By limiting Ca^{2+}-diffusion spines may help to increase input specificity in the brain (Yuste and Denk, 1995; Shepherd, 1996).

Second, spines increase the surface area of dendrites and thus the number of synapses that can be formed per dendritic length (Swindale, 1981). Furthermore, spines help to maximize axonal wiring efficacy by enabling synaptic connections without the axons having to take long-winded routes from one dendrite to the next (Bonhoeffer and Yuste, 2002).

Third, it has been proposed that the narrow spine neck imposes an electrical resistance and thereby amplifies EPSPs in the spine. However, spine neck conductances are much larger than excitatory synaptic conductances and thus are unlikely to act as a resistance for EPSPs (Svoboda et al., 1996). So far, there is no experimental evidence that spines promote electrical compartmentalization.

Spines exist in a variety of shapes, and different morphological criteria have been employed to group different spine types into several categories (Jones and Powell, 1969; Desmond and Levy, 1985; Harris et al., 1992; McKinney et al., 1999). A widely used categorization includes five morphological groups (Sorra and Harris, 2000) (**Fig. 3**):

- Stubby spines (**A**)

  Very short, without a distinguishable neck and head
- Thin spines (**B**)

  Spines with a long neck and clearly visible head (<0.6 µm)
- Sessile spines (**C**)

  Spines with a long neck without a bulbous head
- Mushroom spines (**D**)

  Big spines, with a neck and a very voluminous head (>0.6 µm)
- Filopodia (**E**)

  Very long, thin protrusions without a head, can contain several synapses

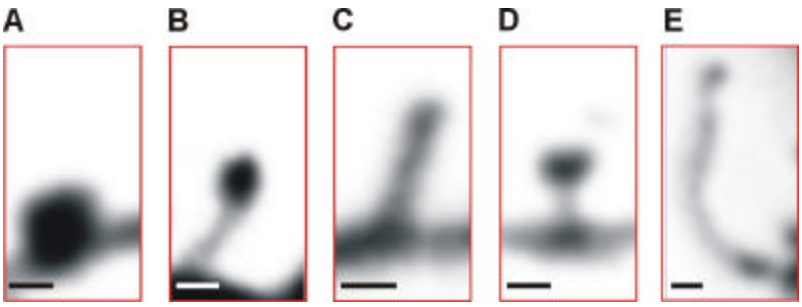

Fig. 3: Morphological groups of dendritic spines (Sorra and Harris, 2000). **A**, Stubby spine. **B**, Thin spine. **C**, Sessile spine. **D**, Mushroom spine. **E**, Filopodium. Scale bars: 1 µm.

It is important to note that this classification might not reflect functional differences between spines. Furthermore, it is conceivable that spines do not belong to a particular class permanently. Given that it has been demonstrated that spines can rapidly change their morphology (Fischer et al., 1998), the different spine morphologies could reflect different stages in the lifetime of a spine.

This pleomorphy confers different spine morphologies with individual characteristics which influence synaptic transmission. Spine size (Korkotian and Segal, 2000), shape (Volfovsky et al., 1999), and neck length (Holthoff et al., 2002) have been reported to influence Ca^{2+} dynamics. Furthermore, spine geometry seems to be critical for AMPAR-expression. AMPARs are primarily expressed in mushroom spines whereas thin spines and filopodia have only few AMPARs (Matsuzaki et al., 2001). Spine volume has also

been found to be proportional to the number of postsynaptic receptors (Nusser et al., 1998) and the number of presynaptic vesicles (Schikorski and Stevens, 1997; Chicurel and Harris, 1992).

These results indicate that spines have an important function in synaptic transmission and that their size and shape effects synaptic efficacy. Thus, modulating spine morphology could be an effective way of mediating synaptic plasticity.

1.4. Morphological plasticity

Morphological plasticity refers to the observation that spines can undergo rapid changes in shape and size. It is particularly prominent during early postnatal development when new synaptic connections between neurons are established (synaptogenesis) and existing ones are refined.

Up until recently technical constraints prevented detailed analyses of morphological stability of neurons over time. In spite of reports demonstrating that the number of dendritic spines can change over time (Globus and Scheibel, 1967; Parnavelas et al., 1973), the morphology of fully differentiated neurons has long been considered to be stable. Only recent advances in cell labeling and imaging have provided the tools for studying morphological stability with a high temporal and spatial resolution, which is essential to reveal subtle changes in spine size, morphology and number.

Different kinds of morphological changes have been observed in young and adult nervous tissue.

1.4.1. Spine development

In rats during the first postnatal week, filopodia have been shown to be abundant and to bear 25% of all synapses. The remaining 75% of the synapses were located on the dendritic shaft itself. Filopodia rapidly protruded and retracted from dendrites (Dailey and Smith, 1996; Ziv and Smith, 1996). With further development the number of filopodia and shaft synapses decreased and thin and mushroom spines started to predominate (Fiala et al., 1998). In young Müller organotypic cultures (one week after preparation), ~40% of all protrusions were filopodia and this number decreased to 1% in four-week old cultures (Collin et al., 1997). During this period the overall density of protrusions increased from 0.4 to 1.2 protrusions/µm. Similarly, it has been shown that *in vivo* the number of filopodia decreased from 12% in one-month old mice to 1% in two-month old animals (Grutzendler et al., 2002). This concordant decrease of filopodia and increase of spines suggests that filopodia represent an early stage in spine formation and that they might mature into spines. Similar results have been obtained by studies overexpressing the postsynaptic density protein 95 (PSD95), which is a major structural component of the postsynapse (Husi et al., 2000; Okabe et al., 2001; Marrs et al., 2001). Keeping in mind potential effects of PSD95 overexpression, these studies demonstrated that synapses initially are formed on filopodia-like processes which transform into mature spines. Furthermore, it has been shown that in organotypic cultures various spine types could originate from filopodia (Parnass et al., 2000). The opposite process (spines turning into filopodia) has also been observed in this study.

In addition, spines can also form directly from the dendritic shaft. Such d*e novo* formation of spines has been reported without transition of filopodia (Okabe et al., 2001; Engert and Bonhoeffer, 1999).

Several hypotheses have been put forward to explain how filopodia might be involved in synapse formation and how a contact to a presynaptic bouton could be established (Harris, 1999). These include active pulling of an axonal branch towards the dendrite or a migration of the axon along a filopodium towards the dendrite.

1.4.2. Transient morphological plasticity

Time-lapse imaging of fluorescence-labeled neurons has revealed that spines exhibit transient shape and size changes on the time scale of seconds (for review, see Bonhoeffer and Yuste, 2002). This rapid motility of spines ('spine wiggling') has first been observed in dissociated hippocampal cultures (Fischer et al., 1998) and was later confirmed in organotypic cultures (Matus, 2000), acute slices (Dunaevsky et al., 1999) and *in vivo* (Lendvai et al., 2000). Although the molecular mechanisms that underlie these shape changes are not clear, they have been shown to require actin polymerization (Fischer et al., 1998; Dunaevsky et al., 1999) and are prevented by AMPAR- and NMDAR-inactivation (Fischer et al., 2000). These results were contributed by the finding that spine motility in dissociated cultures is enhanced by blocking action potentials (Korkotian and Segal, 2001), further suggesting that rapid shape changes are regulated by synaptic activity. However, this dependence could not be confirmed in hippocampal slices, where the blocking of synaptic activity did not increase spine motility (Dunaevsky et al., 1999).

The role of synaptic activity and developmental stage for these transient changes in spine morphology has also been demonstrated *in vivo*. Whisker trimming, which abolishes sensory input into the barrel cortex and leads to reduced neuronal activity, can have a stabilizing effect on spine morphology in the rat barrel cortex during a short critical period ranging from 11-13 days after birth (P11-P13) (Lendvai et al., 2000).

The functional significance of rapid spine motility is still unclear. A possible explanation could be that spines which lack a presynaptic partner wiggle and that they actively search for a presynaptic partner by rapidly changing their shape and size. This idea is supported by a study on dissociated hippocampal neurons demonstrating that spine motility is reduced in spines that contact a presynaptic bouton (Korkotian and Segal, 2001). However, no such relationship between spine motility and contact to a presynaptic partner has been observed in slices from cerebellar Purkinje cells (Dunaevsky et al., 2001).

All in all, spines can rapidly alter their morphology and these changes seem to be regulated by synaptic activity. Therefore, it is likely that spines do not belong to a single morphological category throughout their lifetime but rather switch between them. In

contrast to these short-term changes, persistent changes in spine morphology have also been studied.

1.4.3. Persistent activity-dependent morphological plasticity

Although morphological plasticity of spines seems to be more prevalent in developing neurons, it has also been found in adult neurons. However, until recently, technical constraints prevented studies that would directly correlate morphological with synaptic plasticity. Such studies had to rely on the comparison of different samples of tissue, as imaging of living tissue and thus comparison of the same sample before and after a manipulation was impossible. Furthermore, simultaneously imaging the morphology and recording synaptic plasticity at identified sites was difficult. Therefore, numerous studies aimed to address a potential relationship between unspecific changes in sensory input and changes in spine morphology.

Visual stimulation has been found to lead to increased spine numbers in the visual cortex (Globus and Scheibel, 1967). Conversely, spine loss and subsequent regrowth was observed after deafferentation in the adult hippocampus (Parnavelas et al., 1974). Whereas visual deprivation decreased spine density (Parnavelas et al., 1973), sensory deprivation of the barrel cortex has not been found to influence spine densities. Instead it induced larger spine heads and reduced spine neck lengths (Vees et al., 1998). This has been confirmed by a recent study using two-photon-laser microscopy (TPLM) in the intact, living brain, which demonstrated that *in vivo* sensory deprivation of the mouse barrel cortex by whisker clipping enhanced the turnover rate of spines rather than spine densities (Trachtenberg et al., 2002).

Furthermore, environmental enrichment resulted in the formation of new spines both in adult rats (Rampon et al., 2000) and during juvenility (Comery et al., 1995; Moser et al., 1997). Raising rats in enriched environments led to increased numbers of multi-synapse boutons in the visual cortex (Jones et al., 1997).

Other factors that increase spine numbers in the hippocampus are stress (Shors et al., 2001), the female steroid hormone estradiol (Cameron et al., 1993) and hibernation (Popov and Bocharova, 1992).

Furthermore, learning and memory have been implied in changes in spine morphology. In adult rats increased spine densities have been observed on basal dendrites of CA1 neurons following training of an associative hippocampus-dependent learning task (Leuner et al., 2003). Focusing on a particular set of synapses with multi-synapse boutons yielded evidence that this type of synapse was increased in the motor cortex after acquisition of complex motor skills (Federmeier et al., 1994) and in the rabbit hippocampus after eye-blink conditioning (Geinisman et al., 2001). In addition, spine neck length in honeybees has been observed to shorten after one-trial learning during their first orientation flight (Brandon and Coss, 1982).

On the cellular level, modulation of sensory input and learning affects the level and pattern of synaptic activity. Changes in synaptic activity could, therefore, account for the effects of learning and sensory input on spine morphology. Indeed, synaptic activity has been identified to determine the morphological stability of neurons (for review, see Yuste and Bonhoeffer, 2001).

However, different approaches to elucidate the relationship between synaptic activity and spine morphology have sometimes yielded conflicting results depending on the culture system and/or the age of the animals used. Chronic blockade of spontaneous activity led to a reduction of spines via AMPAR activation in organotypic hippocampal cultures prepared from juvenile rats (McKinney et al., 1999). In line with this, treatment of organotypic hippocampal cultures with tetrodotoxin (TTX) and the NMDAR-blocker 2-amino-5-phosphonovalerate (APV) prevented the age-dependent increase in spine density (Collin et al., 1997). Conversely, other studies reported an increase in spine density soon after blocking synaptic transmission in hippocampal slices (Kirov and Harris, 1999). Spine densities were elevated in hippocampal slices in comparison to perfusion-fixed hippocampi (Kirov et al., 1999), which can also be explained by the reduction of synaptic activity due to lack of synaptic input after slice preparation. Furthermore, NMDA application to individual dendritic segments of dissociated hippocampal neurons, which supposedly results in increased synaptic activation, has been observed to cause spine loss (Halpain et al., 1998).

A possible explanation for these apparently contradictory results could be that the reduction of synaptic activity for only a short period of time leads to an increase in spine numbers, which could act to compensate for the reduced levels of activity. Chronically blocking synaptic activity, however, deprives synapses of activity for extended periods of time, thereby causing loss of spines.

Modulation of synaptic activity is known to induce synaptic plasticity. Therefore, it is reasonable to assume that synaptic plasticity underlies morphological changes. In fact, several studies have confirmed that LTP can induce changes in spine morphology. LTP in the dentate gyrus of the hippocampus has been found to increase spine volume (Van Harreveld and Fifkova, 1975). However, in another study spine volume remained constant and instead, the number of shaft synapses increased and the variability of spines in the CA1-region decreased (Lee et al., 1980).

The development of new imaging techniques, namely confocal microscopy and TPLM, allowed for repetitive or chronic imaging of living tissue. Using these techniques, tracking the fate of a given structure over time has become possible. It could be shown that chemically induced LTP led to changes in spine length and orientation (Hosokawa et al., 1995).

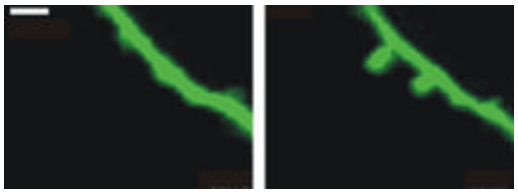

Fig. 4: Formation of dendritic spines after LTP induction. Comparison of a dendritic branch before (left) and 46 min after LTP induction (right); scale bar: 2 µm (from: Engert and Bonhoeffer, 1999).

Time-lapse imaging with TPLM revealed that formation of new spines (Engert and Bonhoeffer, 1999; **Fig. 4**) and filopodia (Maletic-Savatic et al., 1999; **Fig. 5**) can be induced by LTP. Their results show that activity-induced spine formation can occur as early as 20-30 min after LTP-induction. It is important to note, however, that it is still unclear whether the new spines and filopodia contain or acquire functional synapses.

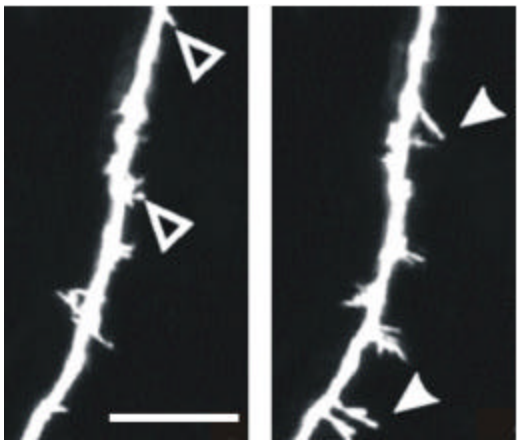

Fig. 5: Formation of filopodia after LTP induction. Filopodia are formed *de novo* (closed arrowheads) 25 min after tetanic stimulation (right), other protrusions retract (open arrowheads); scale bar: 10 µm (from: Maletic-Savatic et al, 1999)

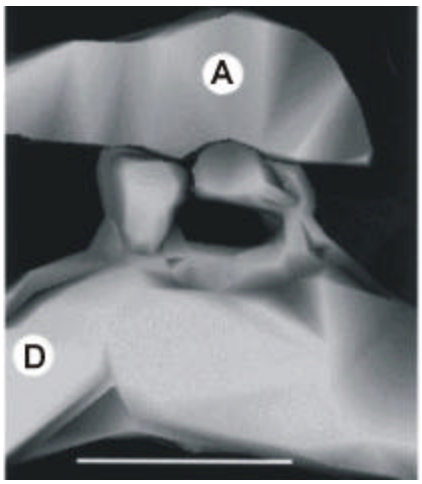

Fig. 6: Electron microscopy image of a multisynapse bouton contacted by two spines; scale bar: 1 µm. A: Axonal bouton, D: Dendrite (from: Toni et al, 1999).

In another study using electron microscopy it was demonstrated that synapses which have previously been potentiated develop perforated postsynaptic densities (Buchs and Müller, 1996). In addition, they tend to form synapses with multi-synapse boutons (Toni et al., 1999; **Fig. 6**). This indicates that new spines are formed or existing ones are recruited to previously potentiated synapses.

To explain how new spines can be generated in mature neurons a model has been proposed which involves spine 'splitting'. Splitting of spines is thought to include the development of a long, narrow protrusion of the dendritic surface (spinule) protruding into the presynaptic bouton. The spine head progressively splits into two, forming a transition state of a branched spine with two heads. The splitting continues until two completely separated daughter spines are generated. Although branched spines are rare and make up only ~10% of all synapses in hippocampal area CA3 (Chicurel and Harris, 1992) there is evidence that increasing synaptic activity can cause an increase in the abundance of branched spines (Jones et al., 1997). The validity of this model is still debated, however, as it has been shown that branched spines never contacted the same presynaptic site and most spinules protruded towards boutons that were not presynaptic to the spine (Sorra et al., 1998; Ostroff et al., 2002).

Considering this, it is not clear whether branched spines indeed represent intermediate stages of a spine in the process of splitting. Furthermore, other mechanisms of spine

formation are also conceivable, e.g. formation directly from the dendritic shaft (Harris, 1999).

In contrast, direct evidence for morphological changes in spines induced by LTD is missing. Application of the NMDAR agonist NMDA (20 µM, 3 min) has been shown to induce long-lasting synaptic depression (Lee et al., 1998). This chemically-induced LTD occludes electrically-induced NMDAR-LTD and therefore probably shares common expression mechanisms. It has been reported that inhibition of calcineurin, which is required during NMDAR-LTD, blocks a decrease in spine number after application of NMDA (10 µM, 5 min; Halpain et al., 1998). Similarly, reduction of spine length has been observed in dissociated hippocampal neurons during prolonged exposure (4 h) to brief pulses of NMDA (20 ms; Segal, 1995).

In these studies induction of NMDAR-LTD and spine retraction has been achieved by different application paradigms of NMDA and a direct link between NMDAR-LTD and spine retraction has not been made. Nevertheless, the results suggest that NMDAR-LTD can cause a reduction in spine size and number.

Taken together, the level of morphological changes in spines is highest during early postnatal development. Nevertheless, the number and morphology of dendritic spines can also undergo rapid changes during adulthood. Morphological plasticity appears to be regulated by synaptic plasticity and it has been suggested that synaptic potentiation induces formation of new spines. Whether, in reverse, synaptic depression causes retraction of spines is less clear.

1.4.4. Summary of aims

There is strong experimental evidence showing that synaptic transmission and spine morphology can be plastic. Changes of spine morphology have been suggested to mediate synaptic plasticity, which is generally believed to be a cellular mechanism for learning and memory. Furthermore, morphological changes have been directly implicated in different forms of learning and memory. Together, this indicates that learning and memory may involve structural changes of spine morphology.

Although potentiation of synapses during LTP has been demonstrated to induce formation of spines and filopodia, the evidence for morphological changes induced by synaptic depression during LTD is less convincing.

The present study aimed to address the question whether LTD can induce morphological changes of dendritic spines. To assess the general stability of spines in the neuronal culture system used for the experiments, the morphological plasticity of unstimulated neurons at different ages was analyzed. The effect of LTD on spine dynamics was investigated by comparing the morphology and number of spines before and after the induction of either NMDAR- or mGluR-LTD. Additionally, the relationship between morphological plasticity and protein synthesis was studied.

2. Methods

2.1. Preparation of Gähwiler organotypic slice cultures

The age of the animals at the time of culture preparation is critical for the quality of the cultures and best results have been reported from using out bred Wistar rats at the age of postnatal day 5-6 (P5-P6) (Gähwiler et al., 1998). The animals were decapitated, scalped, and the underlying skull was removed. The brain was gently transferred into a ice-cold drop of preparation solution consisting of Gey's balanced salt solution (GBSS; Gibco) + 50 mM Glucose + 1 mM kynurenic acid (pH = 7.2) (**Fig. 7**). The dissection of the hippocampus followed standard procedures as used for the preparation of acute hippocampal slices. The hippocampi were then cut into 400 µm thick sections with a tissue chopper (McIlwain, Mickle Lab. Eng., Gomshall, England) using ethanol-cleaned razor blades (Fine Science tools, Heidelberg, Germany). If necessary adjacent sections were carefully separated from each other and transferred to a Petri dish with preparation solution and stored at 4 ºC for 30-60 min.

Afterwards, individual slices were placed on sterilized glass cover slips (Kindler, Freiburg, Germany) and submerged in a drop of 20 µl centrifuged (2500 rpm, 4 ºC, 20 min) chicken plasma (Cocalico Biologicals, Reamstown, PA, USA). The drop was dispersed over the whole cover slip with a small spatula and 30 µl thrombin (Merck, Darmstadt, Germany) were added (50-100 U/ml). The cover slips were then kept at room temperature for 20 min for the plasma to coagulate, before they were transferred into cell culture tubes (Nunc, Wiesbaden, Germany) and 750 µl of culture medium (for composition, see page 34, **Table 1** and **Table 2**) were added to each culture tube. The cultures were kept in a specially designed incubator (Schütt Labortechnik, Göttingen, Germany) at 35 ºC in which roller drums ensured a continuous rotation of the tubes (10 revolutions/h). Thereby, the cultures were half the time submerged and half the time exposed to air.

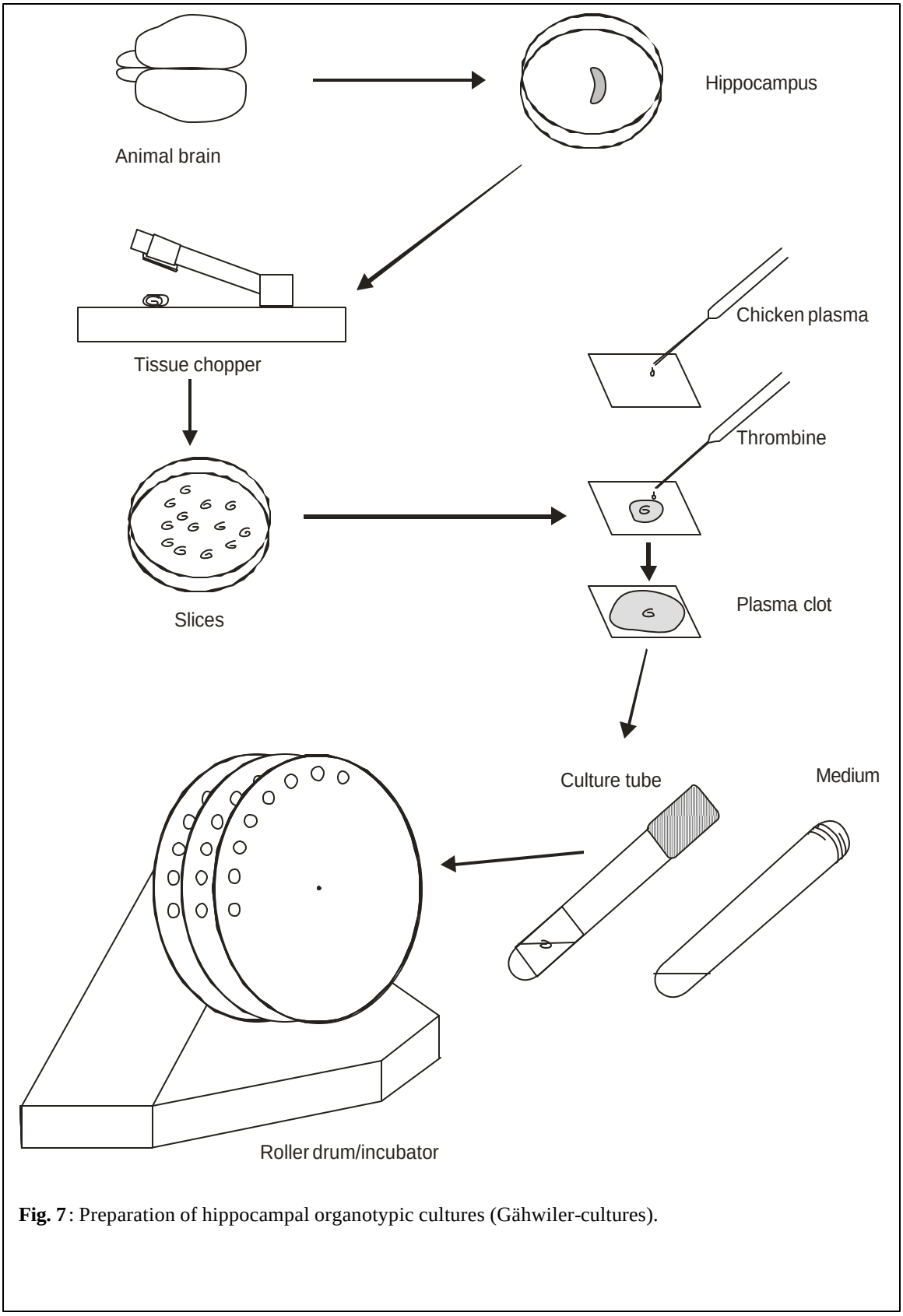

Fig. 7: Preparation of hippocampal organotypic cultures (Gähwiler-cultures).

Three days after preparation, 1 µM of each of the mitosis inhibitors cytosine ß–D-arabinofuranoside, uridine, and 5-fluoro-2'-deoxyuridine were added to the medium. 24 h later, the medium was replaced with inhibitor-free medium. Replacement of culture medium was carried out once a week. The slices were maintained in culture for at least 14 days (days in vitro; DIV) prior to experiments.

2.2. Two-photon-laser microscopy

Since its development in the early 1990s (Denk et al., 1990), two-photon-laser microscopy (TPLM) has been proven to be a potent tool in investigating neuronal morphology in living tissue over time. Therefore, it was also the method of choice to study morphological plasticity of spines in the present study.

TPLM depends on the principle of fluorescence, which involves excitation of a molecule to higher energy levels by light of a specific wavelength ($?_{ex}$) and emission of light with a longer wavelength ($?_{em}$) (**Fig. 8**). In TPLM the fluorophore is excited with light which has twice the wavelength of its excitation optimum (**Fig. 8**). The summation of the excitation energy of two photons (two-photon effect), each of them possessing only half of the necessary energy, leads to emission of fluorescent light. Under the condition of very high photon densities the probability of two photons exciting a fluorophore molecule simultaneously is increased. However, this condition is only met in the focal point of the objective, as the light beam is maximally compressed in space. Consequently, in TPLM the generation of fluorescence signals is limited to the focal point of the microscope.

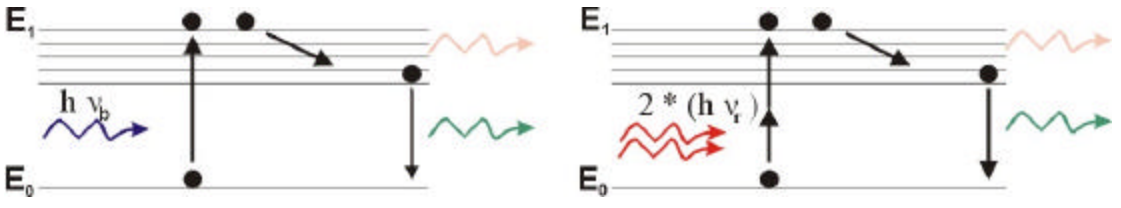

Fig. 8: Jablonski-diagram of the two-photon effect.
The horizontal black lines denote different energy levels (E_0, E_1) of electrons (black dots). Colored arrows represent single photon excitation (blue), two-photon excitation (red), fluorescent light (green), and thermal emission (orange).

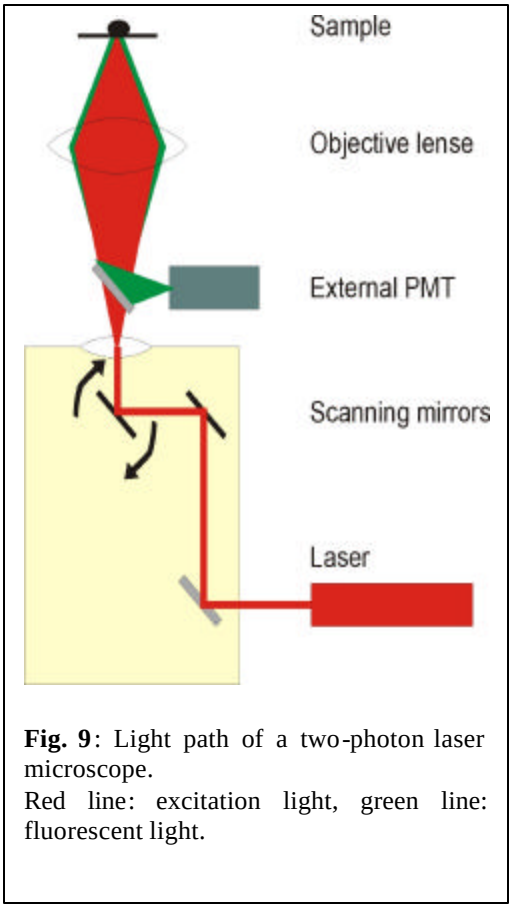

Fig. 9: Light path of a two-photon laser microscope.
Red line: excitation light, green line: fluorescent light.

In general, the resulting two-photon excitation spectra are broader in comparison to one-photon excitation spectra. Therefore, most fluorescent dyes can be excited over a broad range of different wavelengths with TPLM. To achieve high photon densities, pulsed lasers are used, concentrating the light to brief pulses (~100 femtoseconds). The intensity within these packages is extremely high (~50 kW), yet the overall intensity is much lower as the pulses are separated by longer periods of time without light.

In TPLM, laser light is used for excitation. The laser beam is directed through a scanning system that scans the laser over a defined area of the sample. The laser then passes through a conventional microscope which directs the scanning beam onto the sample. The emitted fluorescent light is collected by the objective and directed through the microscope to a photo detector (photomultiplier tube, PMT; **Fig. 9**).

TPLM has several advantages over conventional fluorescence or confocal microscopy, which make it especially suited for collecting high resolution images of living cells over time:

- Reduction of phototoxicity.

  As only fluorophores within the focal spot are excited, the amount of bleaching and phototoxicity caused by activated fluorophores is minimized.
- Increased signal sensitivity.

  As the fluorescent light is produced in only one plane, out-of-focus light is not an issue. Stray light can also be collected efficiently by the external PMT in close proximity to the sample. Consequently, weaker signals can be detected in TPLM.

- Increased penetration depth.

  Because the two-photon-effect requires longer wavelengths to excite a given fluorophore, infrared light is used for excitation. At this wavelenths light is less damaging and can penetrate deeper into biological tissue. Therefore, TPLM has a higher penetration depth than confocal microscopy.

2.3. Technical data of TPLM

The laser light used for excitation of fluorophores was produced by a laser system (Millenia) from SpectraPhysics (Mountain View, CA, USA). In this system a diode laser of 20 W is fiber-coupled to an all solid-state laser (Neodymium yttrium vanadate, Nd:YVO_4, ?= 532 nm). The solid-state laser was tuned from 4.5-5 W in intensity to pump an infrared-laser (Tsunami), the light of which was then used as excitation light source for TPLM. The Tsunami is a pulsed Ti:sapphire laser which produces laser pulses of 80-130 fs at 80 MHz.

To label neurons, two different fluorescent probes were used. For LTD experiments in cultures aged between 14 and 30 DIV, calcein was used as fluorescent indicator ($?_{ex}$= 490 nm, $?_{em}$= 509 nm). To image calcein-labeled neurons with TPLM, the laser was tuned to 840 nm.

To label younger cultures (4-11 DIV) and to test protein expression neurons were transfected with enhanced green fluorescent protein (eGFP: $?_{ex}$= 495 nm, $?_{ex}$= 517 nm). Two-photon excitation of eGFP at 890 nm produced sufficient fluorescence to image dendritic spines.

In order to avoid photobleaching and thermal heating of the probe by the laser light, its intensities were minimized. The output power of the Tsunami was measured with a laser power meter (PM500A; Molectron, Portland, OR, USA) and was in the range of 450-500 mW. This output power was attenuated by neutral density filters to 9-15 mW before reaching the objective of the microscope. The attenuation of the laser light by the objective was difficult to measure because the working distance of the objective was very small. The laser beam diverged considerably from the focus point and its intensity could

not be measured reliably. For signal detection an external photomultiplier (PMT) was used (R-3896; Hamamatsu, Herrsching, Germany).

2.4. Description of the experimental setup

The experimental setup combined components for electrophysiological recordings and image acquisition by TPLM. The whole setup, including the recording chamber, the scan head of the TPLM, and the lasers were mounted on a vibration-free table (Melles Griot, Bensheim, Germany). The recording chamber consisted of V4A-steel and allowed continuous perfusion with a recording medium via an application and a suction tube. The suction tube was connected to a metal canula, the tip of which was designed to minimize medium movements and surface perturbations due to unstable suction. Both tubes were connected to a peristaltic pump (minipuls 2, Abimed, Düsseldorf, Germany) which allowed for the continuous perfusion of medium at ~ 1 ml/min. In addition, the recording chamber was equipped with an indifferent electrode (Ag/AgCl-pellet; Science Products, Hofheim, Germany), a heating sensor and a heating wire for temperature control (Mawitherm, Monheim, Germany). The chamber was held in place by a custom-made platform, mounted onto the microscope. The manipulators for the recording and the stimulating electrodes (Luigs & Neumann, Ratingen, Germany) were also attached stably to that platform.

All images were taken through an inverted microscope (Axiovert 35; Zeiss, Oberkochen, Germany) using a 63x oil immersion objective (Neofluar; Zeiss) with a numerical aperture of 1.25. The immersion oil (518N; Zeiss) had a refractive index N_e = 1.518. Together with the scan head, the microscope was mounted onto two electriconically controlled motors (Newport, Deckenpfronn, Germany), which allowed lateral displacement in X/Y-directions to adjust the field of view.

Via routing mirrors (λ_{refl} = 720-880 nm; Laser Components, Olching, Germany) the laser light was directed into the scan head of a conventional confocal microscope (MRC1024, BioRad, Bristol, UK). A motorized focus control allowed for imaging of defined horizontal sections during image acquisition. After passing through the scan head, the

laser beam was directed into the objective of the microscope via a customized filter cube. The incoming horizontal beam was directed upwards by a low-pass dichroic beam splitter (? $_{trans}$ > 650 nm; AHFanalysentechnik, Tübingen, Germany) which reflected the laser light and transmitted the fluorescent light. The latter was collected by the objective and directed into the PMT. The beam splitter and two more short-pass filters in front of the PMT prevented stray laser light from entering the PMT. The PMT was mounted on a mobile sledge inside the microscope to allow for electronic light detection and naked eye visualization.

2.5. Electrophysiology

All experiments were performed in carbogen-bubbled (95% O_2, 5% CO_2) tyrode solution at 32ºC (composition of tyrode, see below). Synaptic responses in the form of excitatory postsynaptic potentials (EPSPs) were recorded intracellularly from CA1-pyramidal neurons using sharp electrodes. The electrodes were made of borosilicate glass pipettes with a filament (Clark, Reading, England) and were pulled to fine tips with an electrode puller (Sutter Instruments, San Rafael, USA). For intracellular recordings electrodes were back-filled with 100 mM calcein (dissolved in 3 M KCl) and 3 M KCl resulting in electrical resistances in the range of 80-110 MO.

To impale a neuron with an electrode, neurons in the pyramidal layer of area CA1 were approached under visual control and penetrated by briefly overcompensating the electrode capacitance. Successful impaling of a neuron was recognized as a sudden drop in potential and occasional spiking of the neuron. A hyperpolarizing current was applied via the recording electrode to stabilize the cell and helped it to regain its ionic equilibrium. Only cells were used for experiments, which required less than 200 pA of current injection to maintain a stable membrane potential. The membrane potential of CA1 pyramidal neurons under these conditions was between -70 and -75 mV. As soon as stable membrane potentials were reached, the neurons were imaged with TPLM. In most cases, the neuron was already well-filled with calcein after several minutes. The recordings were made with an Axoclamp 2B amplifier (Axon Instruments, Foster City,

CA) in current clamp mode. The recorded potentials were amplified 10 fold and bandpass filtered at 1 Hz and 1 kHz. Electrical stimulation and data acquisition was triggered by an external triggering device (Master8, A.M.P.I., Jerusalem, Israel). The signal was analyzed with custom designed acquisition software (LabView, National Instruments, Munich, Germany). Synaptic responses, membrane potentials, and the currents injected via the recording electrode were recorded.

For extracellular recordings the signal from the recording electrode was amplified 200-fold. The electrophysiological data were recorded and analyzed in such a way that the group identity of the experiment was revealed only after the data had been analyzed.

It has been demonstrated previously that formation of new filopodia can be induced in a restricted part of the dendritic tree by tetanic stimulation if the stimulation electrode is placed close to the dendritic tree (Maletic-Savatic et al., 1999). Placing the stimulation electrode in proximity to a dendritic branch (3-10 µm) resulted in filopodia growth within 30 µm distance to the stimulation electrode. This local stimulation approach was also used in the present study to induce synaptic activation in a dendritic branch close to the stimulation electrode. Although it cannot be excluded that synapses elsewhere were also activated (see Discussion), subsequent analysis of spine morphology was restricted to the area around the site of stimulation (**Fig. 10**).

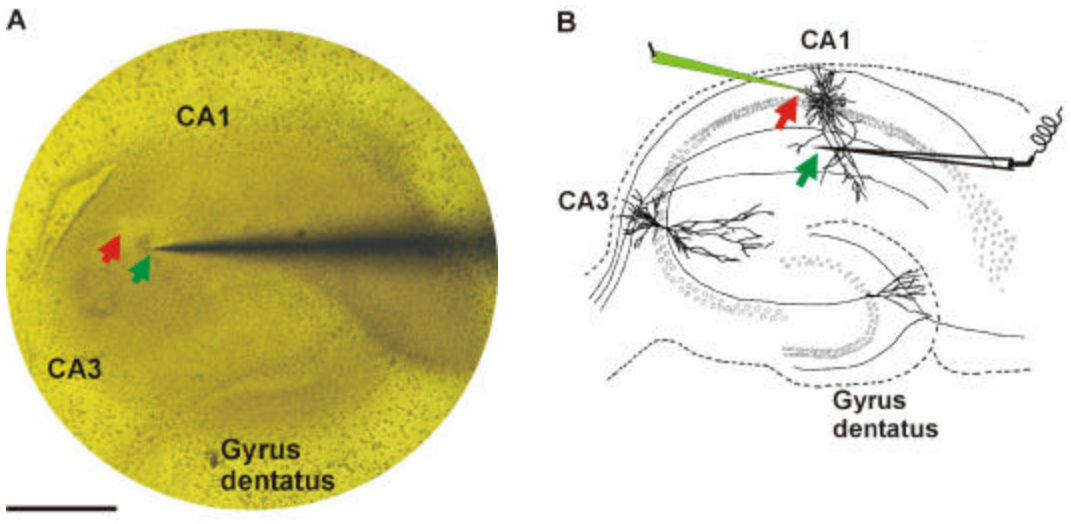

Fig. 10: Transversal section of the hippocampus with recording (red arrow) and stimulation electrode (green arrow).
A, Light microscopic image of a hippocampal organotypic culture (12 DIV; scale bar: 1 mm). **B**, Schematic view of a transversal section of the hippocampus.

The tip of the stimulation electrode was placed on the same horizontal plane as an arbitrarily chosen dendritic branch. To avoid direct stimulation of the dendrite and thereby activate voltage-dependent Na^+ or Ca^{2+} channels, the distance of the stimulation electrode to the closest dendritic branch was kept in the range of 10-30 µm.

To be able to image a neuron and the stimulation electrode simultaneously with TPLM, a glass electrode (Clark Reading, England) filled with Tyrode + 10 mM calcein was used (tip resistance: 4-10 MO). The electrode was placed close to branches of the dendritic tree on the same horizontal plane.

To induce synaptic responses, Schaffer collaterals were stimulated via the stimulation electrode by applying square pulses of 15-40 µA for 50 µs using a stimulus isolator (WPI, Berlin, Germany). The stimulus intensities were adjusted such that the evoked EPSP-size was half-maximal. In some experiments, the neuron continuously fired action potentials in response to the stimulation. In these cases, minimal stimulus intensities were applied, which were just sufficient to evoke action potentials. Baseline values of synaptic transmission were recorded by stimulating at 0.03 Hz for 10-20 min. In the case of stable baseline responses, LTD was induced by applying low-frequency-stimulation (LFS), which is a standard induction protocol for NMDAR-LTD (Dudek and Bear, 1992). LFS consisted of 1 Hz-stimulation applied for 15 min (900 pulses). Afterwards, synaptic responses were again recorded at 0.03 Hz.

During mGluR-experiments baseline stimulation was continuously applied at 0.03 Hz throughout the experiment. To induce mGluR-LTD, 50 µM of DHPG was added to the recording Tyrode solution for 10 min and then washed out with Tyrode solution.

To study the effect of LTD on spine morphology, experiments, in which NMDAR- or mGluR-LTD had been induced successfully, were compared with control experiments. Experiments were included in the LTD group if the mean level of synaptic depression 50-60 min after LFS or DHPG-application was < 90% of the baseline response. The baseline response was calculated as the average EPSP amplitude during 10 min before LFS or DHPG application. The amount of depression could not be quantified in experiments in which the stimulation elicited mainly action potentials during baseline stimulation. These experiments were included into the LTD-group if action potentials were absent after LFS. The results of the LTD-experiments were compared with results of experiments in which

LTD was not induced. Experiments in which LFS did not result in a reduction of EPSP amplitude to less than 90% served as control group for the NMDAR-LTD. The control group for mGluR experiments was treated with bath-application of the vehicle solution of DHPG (50 µM NaOH in recording Tyrode, no DHPG), which did not have any effect on the EPSP size.

To study the protein synthesis dependence of NMDAR- and mGluR-LTD, Schaffer collaterals were stimulated in area CA1 and field excitatory postsynaptic potentials (fEPSPs) were recorded with extracellular electrodes that were positioned in the pyramidal cell layer of CA1. Extracellular electrodes were back-filled with 3 M NaCl resulting in resistances of 5-20 MO.

The average EPSP amplitude 50-60 min after LFS or DHPG application was calculated relative to the average EPSP amplitude 10 minutes before the LTD-induction stimulus. Deviation from the mean was expressed as standard error of the mean (SEM).

2.6. Image acquisition and analysis

Laser intensities were optimized such that an optimal signal-to-noise ratio was achieved. With the laser intensities typically used for experiments, detrimental effects due to exposure to laser light on neuronal morphology and electrophysiological behavior were never observed. Repetitive imaging over several hours and prolonged exposure to laser light during acquisition of large three-dimensional images did not cause changes of membrane potential, synaptic responses, or morphological changes.

Image acquisition was performed using LaserSharp software (BioRad). Images were acquired with maximal possible exposure time (~3 µs/pixel) and averaged using a Kalman filter (n = 2). Neurons were imaged with zoom factors varying from 1.5-2.8. As image dimensions were in the range of 116 × 116 µm - 62 × 62 µm (1024 × 1024 pixels) in x/y-direction (horizontal) the pixel resolution ranged from 0.110 µm/pixel - 0.061 µm/pixel. In the z-dimension (vertical) the step size between individual image planes was 0.3-0.6 µm. Three-dimensional images consisted of images of different horizontal planes ranging from ~10 µm above to ~10 µm below the plane of the

stimulation electrode resulting in 40-60 sections/image (the whole dendritic tree of a neuron in the organotypic cultures typically spanned ~50 µm in vertical dimensions).
Image analysis was done using Confocal assistant (BioRad) and Imaris (Bitplane, Zürich, Switzerland) software without further image processing. In order to detect morphological changes, the images were analyzed by eye in a section-by-section fashion, allowing visualization of all the spines protruding roughly on the same plane as the image section. The spine morphology from single sections was compared between different images. Two-dimensional projections were only used to document the results because small structures can easily be obscured when the maximum signal from all sections is condensed into a single two-dimensional image.

In order to quantify morphological changes, spines were grouped into four categories (cat) according to their size (Engert and Bonhoeffer, 1999):

- Cat 1
  Small, stubby spines
- Cat 2
  Middle-sized spines
- Cat 3
  Long, thin spines or mushroom spines
- Filopodia
  Long (> 5 µm), thin processes, without a clear head

Image analysis was performed by a neutral observer who was unaware of whether images belonged to the LTD- or control groups. For every experiment an image before LFS was compared with at least one image after LFS. As no three-dimensional reconstruction was performed, spines protruding perpendicular to the image plane remained above or below the dendrite and were often hard to distinguish from the dendritic branch in front of or behind them. Therefore, only spines that protruded roughly in parallel to the imaging plane were analyzed.
All spines which could be unambiguously identified were included in the analysis and their morphology was compared between images taken before and 60 min after LTD

induction. Per definition, a spine was considered to have changed in morphology if it switched one (e.g. 0? 1, 1? 2, 3? 2, 1? 0) or two and more cat (e.g. 0? 2, 1? 3, 0? 3, 2? 0). These changes are referred to as 1-cat and 2-cat changes, respectively. The categories of all visible spines, their x/y-coordinates and overall spine number were noted.

Spine densities of individual neurons were determined by counting all visible spines and measuring the overall length of the analyzed dendrites in a two-dimensional projection. Measurements of dendritic length were done using MetaMorph (Universal Imaging, Downingtown, PA, USA)

2.7. Statistics

To be able to compare 1-cat spine changes between NMDAR-LTD and control experiments, an analysis of variance (ANOVA) was used to detect significant differences between the groups. The numbers of one-cat spine changes during mGluR-LTD and 2-cat changes during mGluR- and NMDAR-LTD were too low to be statistically analyzed.

Parametric tests could not be applied to compare the levels of LTD in the presence or absence of protein synthesis inhibitors. They consider the actual value of the means to compare and require a normal distribution of the data. Because the number of experiments for the different groups was small, a normal distribution could not be assumed. Therefore, an ANOVA was used to compare the mean EPSP amplitudes between cycloheximide, anisomycin, and control experiments after NMDAR-LTD induction. The nonparametric Kolmogorov-Smirnov-test was applied to compare the anisomycin and control group after mGluR-LTD induction.

2.8. Chemical solutions and Drugs

Calcein

The highest solubility of calcein (Sigma, Taufkirchen, Germany) in water is at pH= 9. Therefore, 1 M NaOH was added while dissolving 100 mM calcein in 3 M KCl under continuous stirring. Once all the calcein had dissolved the pH of the solution was adjusted to 7.2 by adding 1 M HCl. Next, the solution was filtered and stored at 4 ºC until use.

Anisomycin/Cycloheximide

Anisomycin and cycloheximide (Sigma, Taufkirchen, Germany) were dissolved in DMSO and stored as stock solutions of 25 mM each at -20 ºC. For experiments each drug was diluted 1:1000 when added to the Tyrode solution resulting in a final concentration of 25 µM of anisomycin or cycloheximide. In the control experiments 0.1% of DMSO was added to the Tyrode solution. The cultures were preincubated in the respective solution for ~ 60 min before baseline recording started.

To test whether anisomycin can diffuse into Gähwiler-cultures, eGFP-adenovirus-infected cultures were incubated in 25 µM of anisomycin overnight.

(RS)-3,5- Dihydroxyphenylglycine (DHPG)

DHPG (Tocris, Bristol, England) was diluted in equimolar concentrations of NaOH and stored as stock aliquots of 100 mM at -20ºC. DHPG was further diluted to 50 µM in Tyrode solution and bath-applied for 10 min to induce mGluR-LTD. Application of DHPG in NaOH did not affect the pH of the recording solution.

Recording solution (Tyrode)

Substance	Concentration [mM/l]
NaCl	137.0
$NaHCO_3$	11.6
$NaH_2Po_4 \cdot H_2O$	0.4
KCl	2.7
$CaCl_2 \cdot 2\ H_2O$	2.8
$MgCl_2 \cdot 6\ H_2O$	2.0
Glucose $\cdot H_2O$	5.6

Table 1

Culture medium for organotypic hippocampal cultures

Substance	Volume [ml]
HBSS	25
BME	50
Horse serum	25
Glucose	1 (33.3 mM)
L-Glutamine (200 mM)	0.5 (0.1 mM)

Table 2

3. Results

The aim of this study was to investigate whether LTD can cause morphological changes of dendritic spines. For this, two forms of LTD (NMDAR- and mGluR-LTD) were induced in neurons of organotypic Gähwiler cultures while their morphology was imaged with TPLM. Images were taken before and after LTD induction and the morphology of spines at these time points was analyzed.

It has been shown previously that various factors can influence morphological plasticity, including the age of the neuronal culture. Therefore, to exclude age effects on spine plasticity in Gähwiler organotypic cultures, spine morphology was studied at different time points after culture preparation. Cultures that exhibited morphological stability over time were then used for the LTD experiments.

3.1. Age-dependent morphological plasticity

Although for most of this study calcein injections through an intracellular electrode were used to label individual neurons, this technique was not applicable to very young cultures. To circumvent this problem, neurons were transfected with the pGFP-N1 plasmid (Clontech, USA) using the gene gun method (Helios gene gun system, BioRad, Munich, Germany). This plasmid contained the eGFP-gene under control of a CMV promoter and led to eGFP expression in transfected cells. One or two days after transfection eGFP levels within neurons were sufficiently high to image neuronal morphology with TPLM. Cultures were maintained in carbogen-bubbled culture medium (+ penicillin/ streptomycin) throughout the imaging experiments to keep the cultures alive during extended imaging sessions (up to 25 h).

Fig. 11 and **Fig. 12** show images of two different neurons, which were transfected with eGFP at 2 DIV and were imaged at 4 DIV. At this age the dendritic tree was not fully developed and dendritic branches were shorter than at later stages. However, it already exhibited the typical pattern of a pyramidal neuron in CA1 with a principle apical dendrite and several basal dendrites protruding from the soma. Spine-like processes were

sparse and differed from mature spines morphologically. Only stubby and small spines without a spine head could be observed. They probably represented immature protospines, as described previously in Gähwiler cultures during the first days after culture preparation (Dailey and Smith, 1996). In contrast to what has been reported in that study, no filopodia were observed in the neuron of **Fig. 11**. To calculate spine densities, all spines that were visible in an image were counted and the overall dendritic length was measured. In the neuron of **Fig. 11** it amounted to 0.074 spines/µm, which was considerably lower than what has been previously found for 1-day old cultures (0.72 protrusions/µm) (Dailey and Smith, 1996) This could indicate that spine density is first decreased after culture preparation, before it subsequently increases again. The neuron was imaged again after 1 h 30 min to assess its short-term morphological plasticity demonstrating that spine-like processes were highly unstable as small protrusions were formed (blue arrows) and retracted (red arrows) within this period of time (**Fig. 11B**).

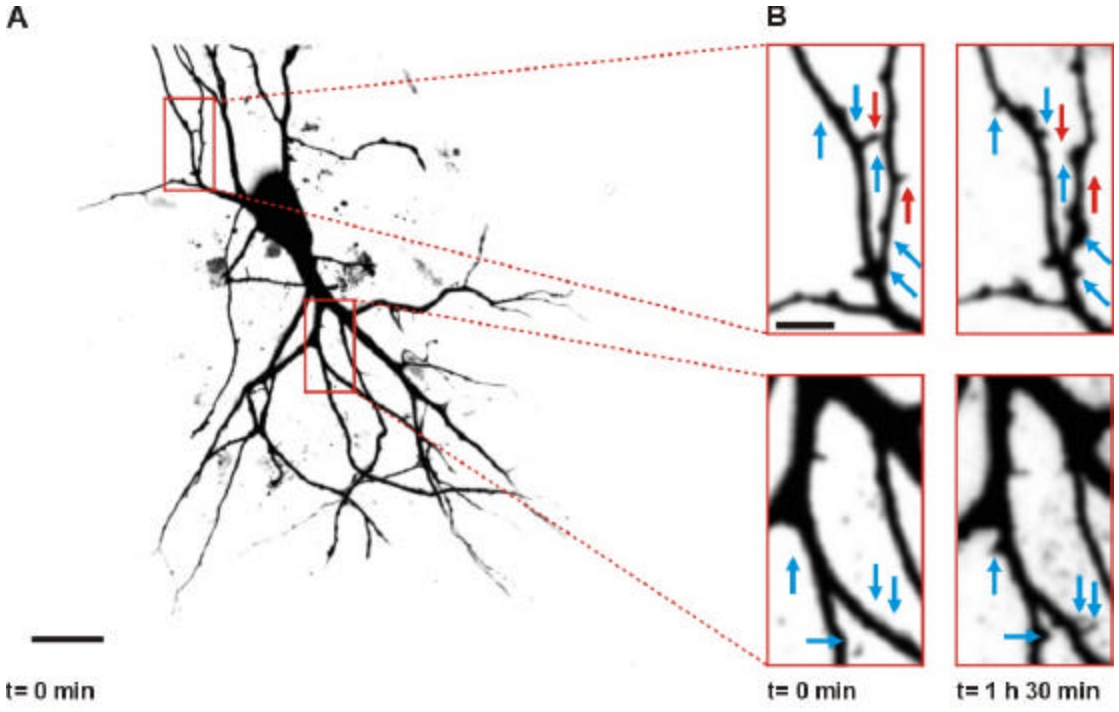

Fig. 11: Examples of morphological short-term changes of a neuron at 4 DIV.
A, Two-dimensional projection of an eGFP-transfected neuron (zoom: 1.0, scale bar: 20 µm). **B**, Magnified images of dendritic regions imaged at the time points indicated (scale bar: 5 µm). Red arrows: retraction of protrusions; blue arrows: growth of protrusions.

The neuron in **Fig. 12** (4 DIV) was imaged during an extended time period (>25 h). The spine density of this neuron was found to be 0.16 spines/µm. Comparison of the two images again showed extensive formation (blue arrows) and retraction (red arrows) of small spine-like processes (**Fig. 12 A**, **B**). In addition, filopodia-like processes were frequently observed to protrude and retract (**C**), confirming previous results from one-week old Gähwiler and Müller cultures (Dailey and Smith, 1996; Collin et al., 1997).

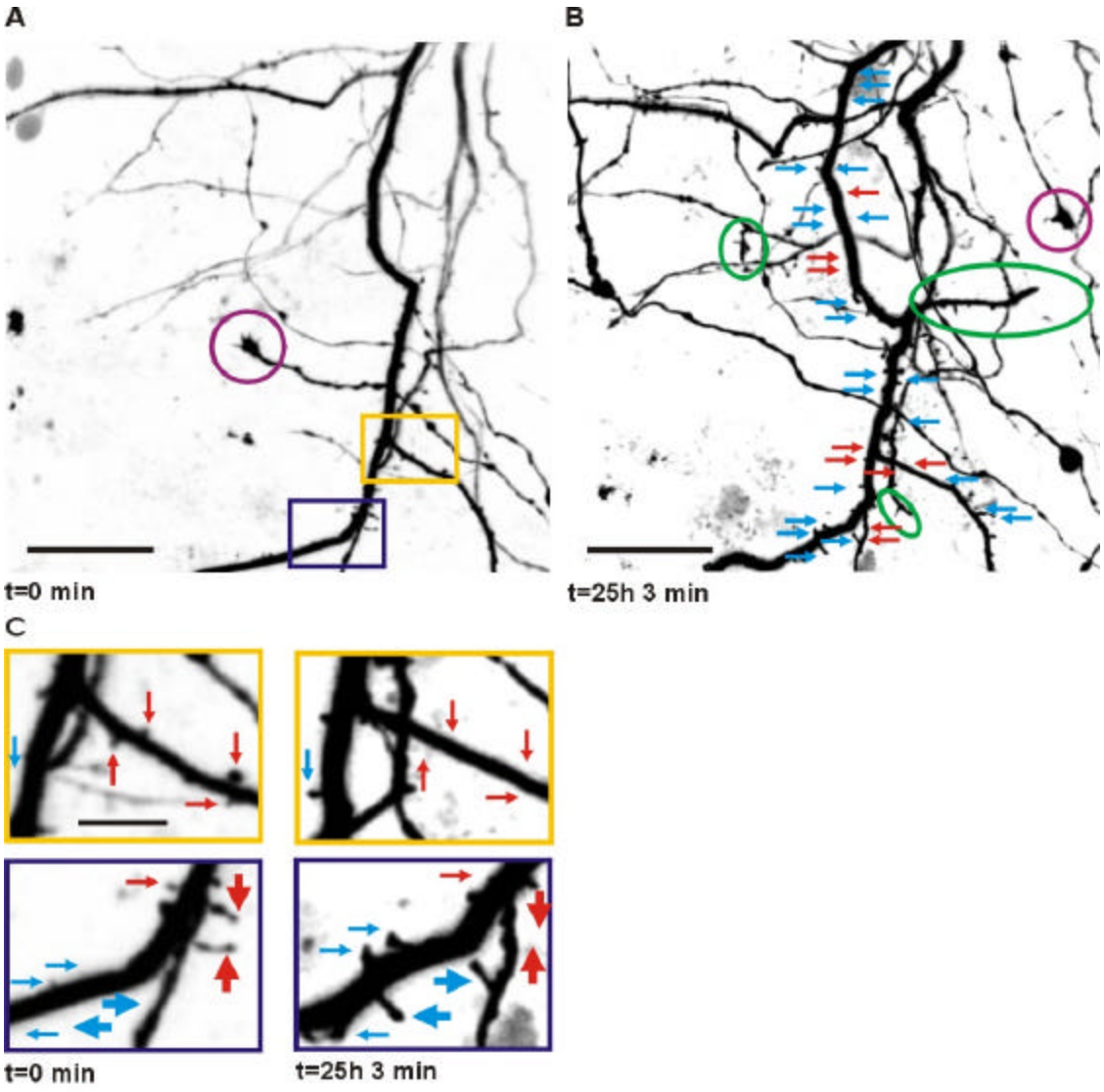

Fig. 12: Example of long-term morphological changes of a neuron at 4 DIV.
A, B, Two-dimensional projections of part of the apical dendritic tree of an eGFP-transfected neuron taken at the time points indicated (zoom: 2.0, scale bar: 20 µm). **C**, Magnified images of two different regions of the dendritic tree indicated by the rectangular of the respective color (scale bar: 5 µm). Purple circle: axonal growth cone; green circle: dendritic growth; thin red arrows: spine retraction; thin blue arrows: spine growth; thick red arrows: filopodia retraction; thick blue arrows: filopodia growth.

Axonal branches could be distinguished from dendrites by their smaller diameter and their varicosities. Being only faintly fluorescent initially, axons increased in fluorescence during the time course of the experiment (**Fig. 12 A**, **B**) due to accumulating amounts of eGFP. In addition to spine growth, in this culture also axonal growth and formation of dendrites could be observed. **Fig. 12 A** shows an axonal growth cone at the leading edge of an axonal branch (purple circle). In **B**, the axon has traversed the field of view. In addition, another axonal growth cone and several examples of newly formed dendritic branches are visible (green circles) confirming previous observations of extensive axonal and dendritic reorganization in one-week old Gähwiler cultures (Dailey and Smith, 1996).

However, the success rate of the gene gun approach was very low, as it predominantly labeled glial cells. The mechanical impact of the gold particles and the air puff also often damaged the cultures. Therefore, only few neurons could be labeled with this method and the results for morphological development in young cultures could not be quantified. Nevertheless, the depicted examples show that early after culture preparation spine densities were low and spines were frequently formed and retracted. Furthermore, spines at this stage did not exhibit the full range of different morphologies of mature spines.

At later stages (>13 DIV) neurons could be impaled with an intracellular electrode. To avoid the damaging effects of the gene gun approach, calcein injections were used to label single neurons. The neuron in **Fig. 13** illustrates the dendritic morphology of an older neuron (14 DIV). As reported previously from Gähwiler and Müller cultures at this age (Dailey and Smith, 1996; Collin et al., 1997), spines exhibited various sizes and shapes and the density of spines (1.12 spines/µm) and dendritic branches was again higher. In contrast to younger organotypic cultures, filopodia were rare.

Repetitive imaging for more than two hours revealed two spines, which changed their morphology for one category (1-cat changes) but no spine changes for two or more cat (2-cat changes). **Fig. 13 B** shows a magnified view of a dendritic branch illustrating incidents of morphological changes, as they were analyzed by the observer. One cat 1 spine disappeared completely (top images) and one cat 3 spine became a cat 2 spine (bottom images). This high degree of morphological stability of spines at this age is in

synchrony with earlier results from 2-week old Gähwiler cultures (Dailey and Smith, 1996).

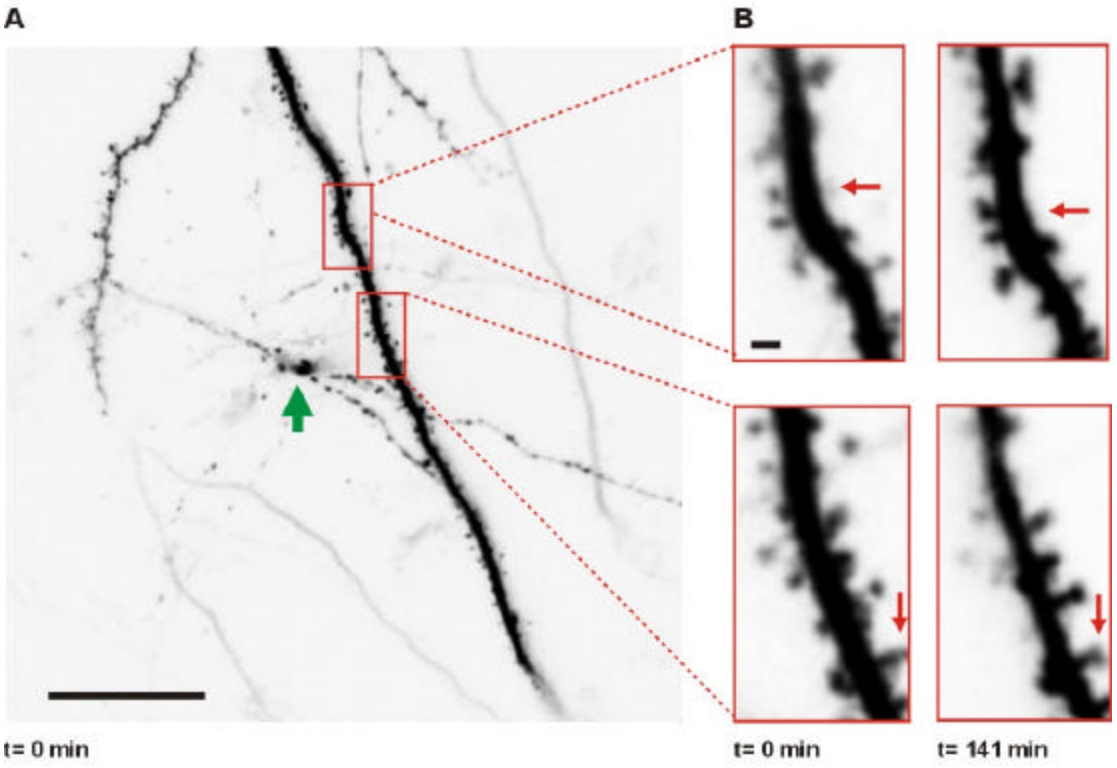

Fig. 13: Example of the morphology of a neuron at 14 DIV.
A, Image of part of the apical dendritic tree of a calcein-labeled neuron (zoom: 2.0, scale bar: 20 µm). Green arrow: tip of stimulation electrode. **B**, Magnified images of two different regions of the dendritic tree (scale bar: 1 µm). Thin red arrows: retraction of spines (one-cat changes).

Given that neurons older than 13 DIV were easily to record from with an intracellular electrode, these cultures were used for the LTD experiments. **Fig. 14** depicts the relationship between spine density and age of cultures between 13 and 30 DIV. The low value of the correlation coefficient (R^2 = 0.030) suggested that spine densities remained constant and did not change with age between two and four weeks.

During this time the average spine density of the neurons was 0.88 ± 0.04 spines/µm (n = 21, 13-30 DIV). It is important to note, however, that spines that protruded perpendicular to the plane of imaging were not analyzed, because they were obscured by the over- or underlying dendritic branch. Therefore, assuming equal distribution of spines in all

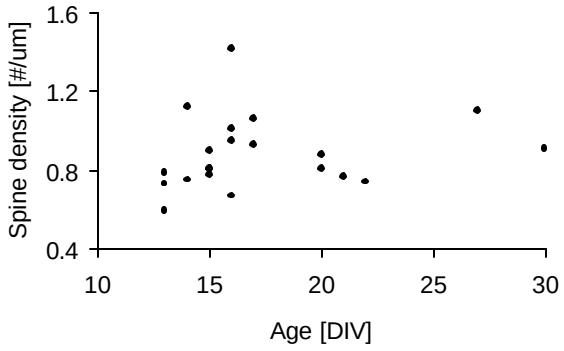

Fig. 14: Relationship between spine density and age of the culture.
No correlation between the two parameters could be observed.

spatial directions, actual spine densities in our cultures were higher than what was measured.

The value for spine density was consistent with previous studies, which reported spine densities to be in the range of 1.0 spines/µm in Gähwiler cultures (14-21 DIV) (McKinney et al., 1999) and 0.9-1.2 spines/µm in Müller cultures (Collin et al., 1997). In comparison, spine densities have been found to be 1.0-1.5 spines/µm in slices from adult rats (Kirov and Harris, 1999), and 2.2 spines/µm in perfusion-fixed three-week old rats (Kirov et al., 1999). However, in living young adult mice (6-10 weeks) the average spine density was 0.4 spines/µm (Trachtenberg et al., 2002) suggesting that the spine density in organotypic cultures and acute slices is higher than *in vivo.*

Considering these results, it could be concluded that during the first two weeks after the culture preparation spine densities were low and spines were frequently formed and retracted. In general, the spines were small and stubby and filopodia were prominent. In comparison, neurons from cultures older than two weeks had high densities of spines and only few filopodia. They exhibited different morphologies similar to what has been reported for mature spines *in vitro* (Dailey and Smith, 1996; McKinney et al., 1999), in perfusion-fixed animals (*in situ*; (Sorra and Harris, 2000) and *in vivo* (Lendvai et al., 2000). Furthermore, spine morphology was highly stable over extended periods of time in these cultures.

These results were consistent with previous findings in area CA3 of the hippocampus where synaptic density was low in organotypic cultures early after culture preparation and subsequent development of synaptic structures paralleled synaptogenesis *in situ* (Frotscher and Gähwiler, 1988; Robain et al., 1994). Thus, within two weeks after culture

preparation pyramidal neurons in Gähwiler cultures acquire many structural features characteristic of age-matched neurons from the intact brain.

Efforts to record intracellularly from neurons of young Gähwiler cultures (< 14 DIV) showed that neurons could not be recorded from at this age. This confirmed previous experiences with intracellular recordings from young organotypic cultures using sharp electrodes (V. Staiger, F. Engert, personal communication). Therefore, only cultures 14-30 DIV were used for studying the effect of LTD on spine morphology. Gähwiler cultures of this age were also used in previous studies measuring synaptic (Debanne et al., 1994) and morphological plasticity (Engert and Bonhoeffer, 1999). Furthermore, pyramidal neurons from cultures at this age have been demonstrated to be fully differentiated and to have dendritic and spine morphologies similar to mature neurons from acute slices (Zimmer and Gähwiler, 1984; Gähwiler et al., 1998) and *in vivo* (Lendvai et al., 2000).

For these reasons cultures with high morphological stability (14-30 DIV) were used to study whether different forms of synaptic depression - NMDAR- and mGluR-LTD - can enhance morphological plasticity of spines.

3.2. NMDAR- LTD

Although it has been reported that LTP can induce morphological changes in Gähwiler cultures (Engert and Bonhoeffer, 1999), it has remained unclear whether LTD would also affect spine morphology in these cultures. To address this question, NMDAR-LTD was evoked by local electrical stimulation.

Axonal fibers of CA3 pyramidal neurons (Schaffer collaterals) were stimulated and synaptic responses were recorded intracellularly from CA1 pyramidal neurons. In a previous study it has been shown that high-frequency tetanic stimulation, which was applied by a stimulation electrode close to a dendritic branch (local stimulation), induced formation of new filopodia within 30 µm distance to the site of stimulation (Maletic-Savatic et al., 1999). Control sites further away on the dendrite (>100 µm) were not affected. The local stimulation approach was also used in the present study to activate synapses in vicinity to the stimulation electrode. In some experiments the stimulation electrode impaled a glial cell or another neuron. In none of the few experiments, in which this happened, dendritic and spine morphology were found to be affected.

Once a neuron was successfully impaled with the recording electrode, the stimulation electrode was positioned to reliably evoke EPSPs. The stimulation intensity was adjusted to induce EPSPs of half maximal amplitude allowing amplitude increases and decreases. Typically, electrical responses ranged from 5-20 mV. In some experiments, however, the stimulation elicited action potentials rather than EPSPs. After recording baseline responses at a very low stimulation frequency (0.05 Hz) for 15-30 min a low-frequency stimulus (LFS: 1 Hz, 15 min) was applied, which is a widely used induction protocol for NMDAR-LTD in slices (Dudek and Bear, 1992) and organotypic cultures (Stoppini et al., 1991). Successful LTD induction was defined as a reduction in EPSP amplitude to less than 90% of the baseline value. Experiments, in which the EPSP amplitude remained more than 90% of the baseline value, were not counted as LTD experiments and served as controls.

Concurrent with the electrical recordings, the morphology of individual neurons was imaged with TPLM. For this, a neuron was labeled with the fluorescent dye calcein (100 mM) via the recording electrode. Because it was not clear at which distances from

the stimulation electrode activation of synapses and therefore morphological changes would occur, the areas around the stimulation electrode were varied between experiments. They covered 3844 - 13456 µm^2 (62 × 62 µm - 116 × 116 µm) depending on the image zoom factor and were roughly centered in respect to the stimulation electrode. Two images were taken during baseline stimulation and only spines that were morphologically stable in both images were included in the analysis. Imaging was continued up to 60 min after LFS at intervals of ~20 min as it has been shown that during LTP morphological changes occur within this time period after LTP induction (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999).

Fig. 15 shows an experiment, in which LFS induced NMDAR-LTD (experiment 5 in **Table 4**). The mean EPSP amplitude 50-60 min after LFS was reduced to 65.4% of the mean amplitude during baseline recording (**Fig. 15C**). **Fig. 16** illustrates an experiment in which LFS did not induce LTD (experiment 2 in **Table 5)**. The mean EPSP amplitude 50-60 min after LFS was 104.9% of the mean baseline response **(Fig. 16C**). In this experiment the stimulation electrode impaled and labeled another neuron or astrocyte (black blob) and an axonal fiber (black arrow) which both started to disintegrate thereafter. Additionally, dendritic morphology (**Fig. 15A**, **Fig. 16A**) and membrane potential (**Fig. 15D, Fig. 16D**) were recorded during the experiments.

The insets in **Fig. 15C** and **Fig. 16C** show averaged EPSP traces before and 60 min after LFS application. The EPSP amplitudes before the LFS were 25.24 ± 0.44 mV and 17.63 ± 0.17 mV, respectively. Recordings of synaptic responses between CA3/CA1 neuronal pairs in Gähwiler cultures revealed that the EPSP amplitude between such a cell pair was ~1 mV (Debanne et al., 1995). Therefore, ~20 Schaffer collaterals of CA3 neurons were stimulated under the stimulus conditions of the present study. The rise times of the EPSPs (time to peak) were 6.98 ± 0.25 ms and 6.94 ± 0.27 ms, respectively, and were similar to what has been found for unitary responses (~6 ms). The duration of EPSPs before LFS was longer than 100 ms, which was considerably longer than has been reported for unitary responses (~50 ms).

This difference in EPSP duration could be explained by a depolarizing effect of inhibitory neurons that were also stimulated. The high [Cl⁻] in the recording electrode could have increased [Cl⁻] within the neurons, resulting in a depolarizing effect of

inhibitory synapses on the recorded neuron. This could prolong the duration of EPSPs. Alternatively, passive membrane properties, e.g. a higher membrane capacity, could also underlie the difference in EPSP duration.

Magnified images of arbitrarily chosen stretches of the dendritic tree illustrate the morphological stability of spines (**Fig. 15B, Fig. 16B**). Colored arrows indicate morphological changes as they were analyzed by an unbiased observer who did not know whether a LTD or control experiment was analyzed. Incidents of 1-cat spine changes are indicated by thin red and blue arrows, respectively. 2-cat changes are indicated by thick arrows. On the shown stretches of dendrite, NMDAR-LTD induction was accompanied by retraction of one spine from category 3 to category 1 (2-cat change), whereas no 2-cat change was observed in the control condition. Several incidents of 1-cat changes were found in both experiments.

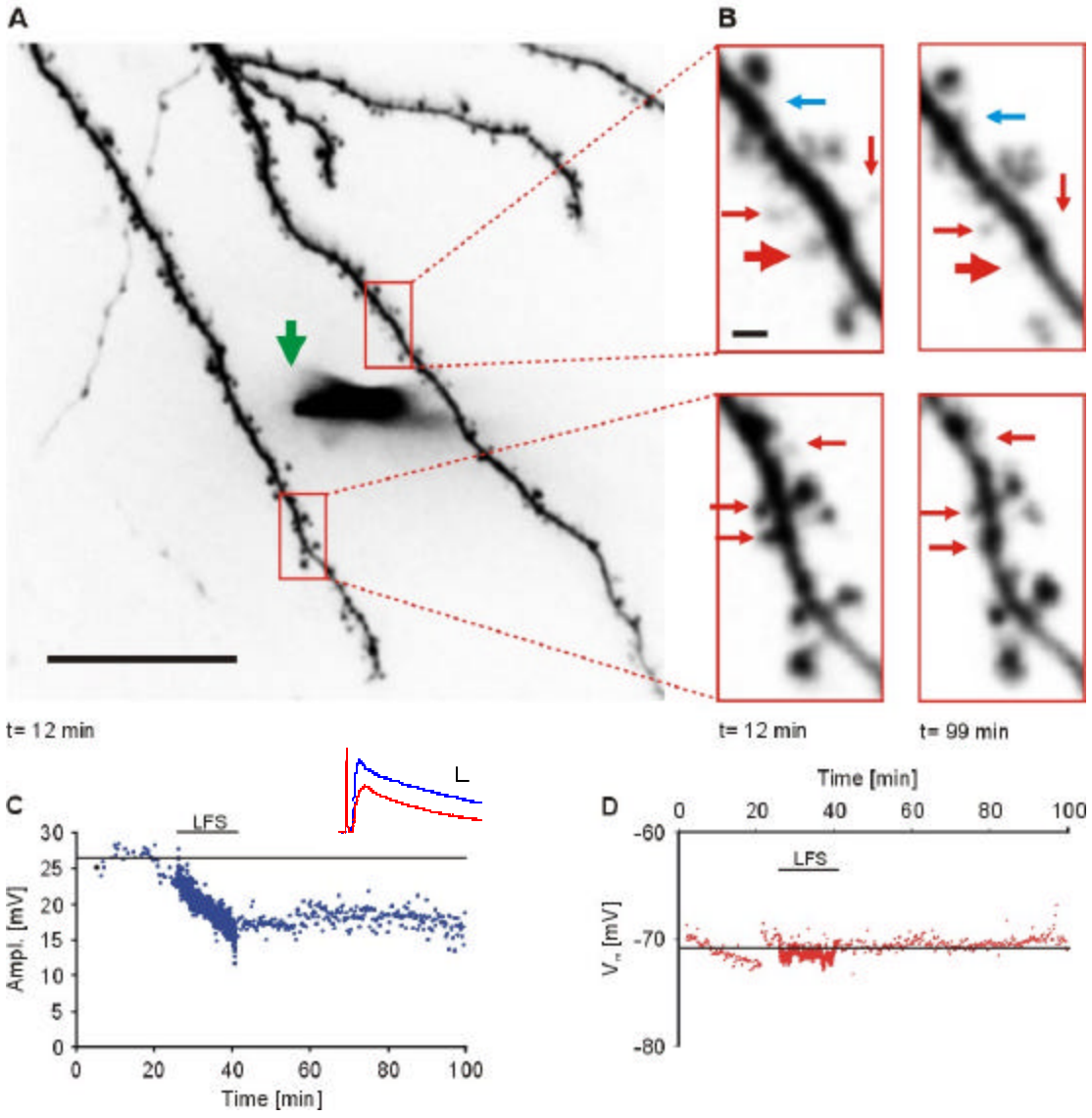

Fig. 15: Example of a NMDAR-LTD experiment (15 DIV).
A, Overview image taken before LFS (zoom: 2.5, scale bar: 20 µm). Green arrow: tip of the stimulation electrode. **B,** Magnified images of two different regions of the dendritic tree imaged at the indicated time points (scale bar: 1 µm). Thin red arrows: spine retraction (1-cat); thick red arrow: spine retraction (2-cat); thin blue arrows: spine growth (one cat). **C**, Time course of EPSPs before and after LFS (t=0 refers to the beginning of the recording). Inset: Averaged traces of 10 EPSPs. Blue: Before LFS, Red: 60 min after LFS (scale bars: 5 mV, 10 ms) **D**, Time course of membrane potential.

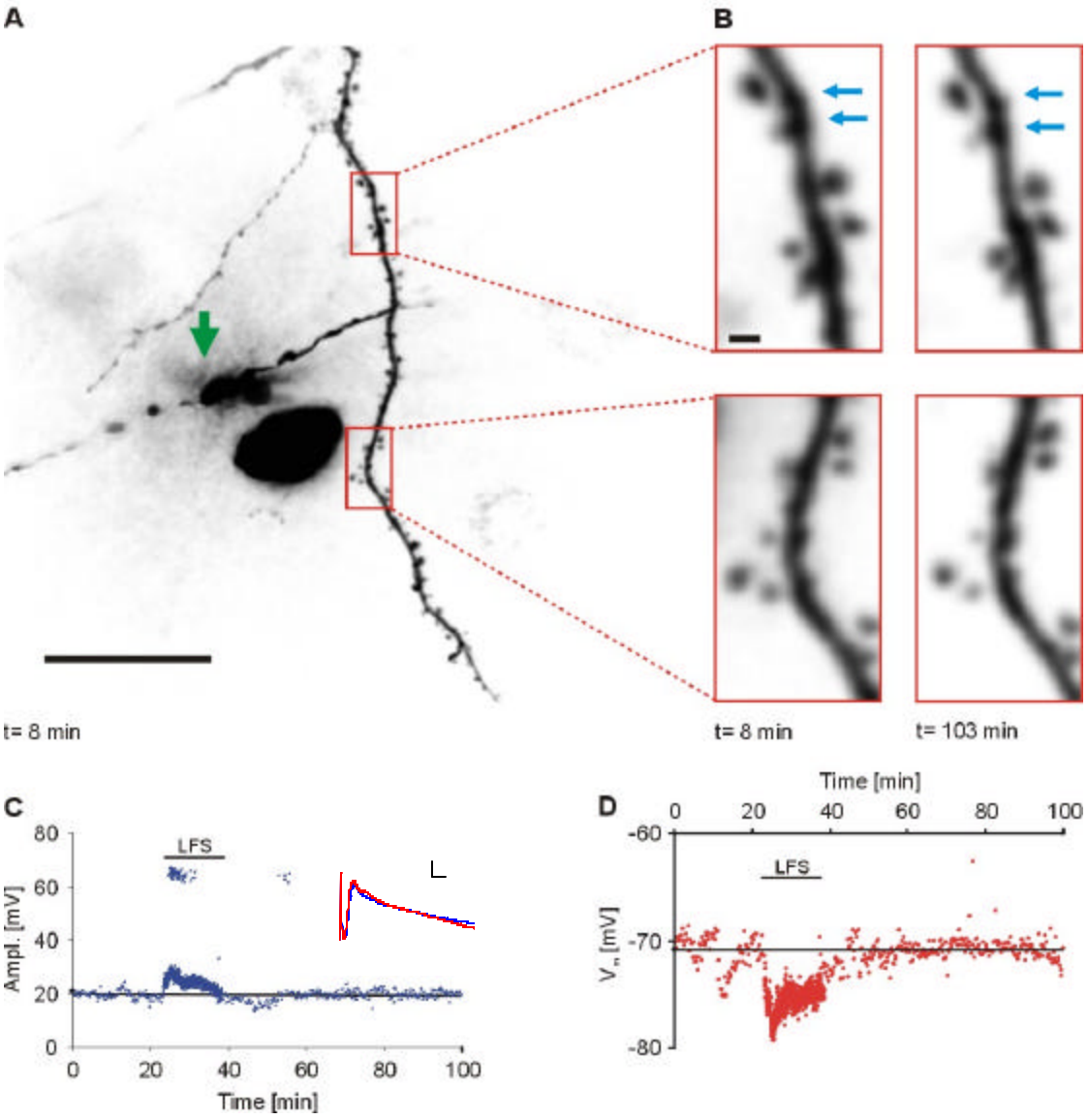

Fig. 16: Example of a NMDAR-LTD control-experiment (21 DIV).
A, Overview image taken before LFS (zoom: 2.2, scale bar: 20 µm). Green arrow: tip of the stimulation electrode. **B,** Magnified images of two different regions of the dendritic tree imaged at the indicated time points (scale bar: 1 µm). Thin red arrows: spine retraction (1-cat); thin blue arrow: spine growth (1-cat). **C**, Time course of EPSPs before and after LFS (t=0 refers to the beginning of the recording). Inset: Averaged traces of 10 EPSPs. Blue: Before LFS, Red: 60 min after LFS (scale bars: 5 mV, 10 ms). **D**, Time course of membrane potential.

The membrane potential was recorded as an indicator of the viability and the electrophysiological condition of the neuron. Only neurons were included in the analysis that exhibited stable membrane potentials during the course of the experiment.

Applying the 90%-criterion for successful LTD induction, the probability of inducing NMDAR-LTD was 67% (n=21). In the LTD-group (n=14), the mean EPSP amplitude 60 min after LTD induction was 66.8% (±4.9%) of the baseline EPSP amplitude. However, six of these neurons fired action potentials during baseline recordings and the amount of depression could not be quantified. They were counted as LTD experiments because after LFS no action potentials were generated any longer.

In the control group (n=7), LFS the mean EPSP amplitude was 104.0% (±3.0%). In three of these experiments the neurons continued to fire action potentials during baseline recordings and after LFS and the amount of depression was not quantified.

On the basis of this classification, the total number of morphological changes and the average number of changes per experiment could be calculated and compared between the LTD and the control group.

3.2.1. NMDAR-LTD and 2-category spine changes

To study the effect of NMDAR-LTD on spine morphology, images from stretches of dendrite before and after LTD induction were compared. To be able to quantify spine changes, spines were grouped into four categories (cat 1-3, filopodia) according to their size (see Methods). The abundances of the different spine categories were not analyzed in detail but it was immediately apparent that filopodia were by far the rarest category. They were unstable over time and never persisted throughout an entire experiment.

Changes in spine morphology were counted as 2-cat changes if a spine changed for two or more categories. **Fig. 17** shows typical examples of 2-cat (thick arrows) and 1-cat changes (thin arrows) of spine morphology as they were analyzed by the observer.

The examples illustrate two major difficulties in analyzing morphological changes, which had to be accounted for during the analysis. Comparison of images of two different time points showed that the fluorescence intensities from a labeled neuron changed during the course of an experiment. This was probably due to the continuous diffusion of calcein

from the electrode into the cell and led to higher fluorescence intensities in images from later time points. Consequently, spines and dendrites often appeared to be larger in later images.

Furthermore, spines were grouped into different categories according to subjective criteria. Especially spines that were of intermediate size were prone to be grouped into different categories by different observers. It was important, therefore, that the entire analysis was done by a single observer who did not know about the identity of the analyzed experiment.

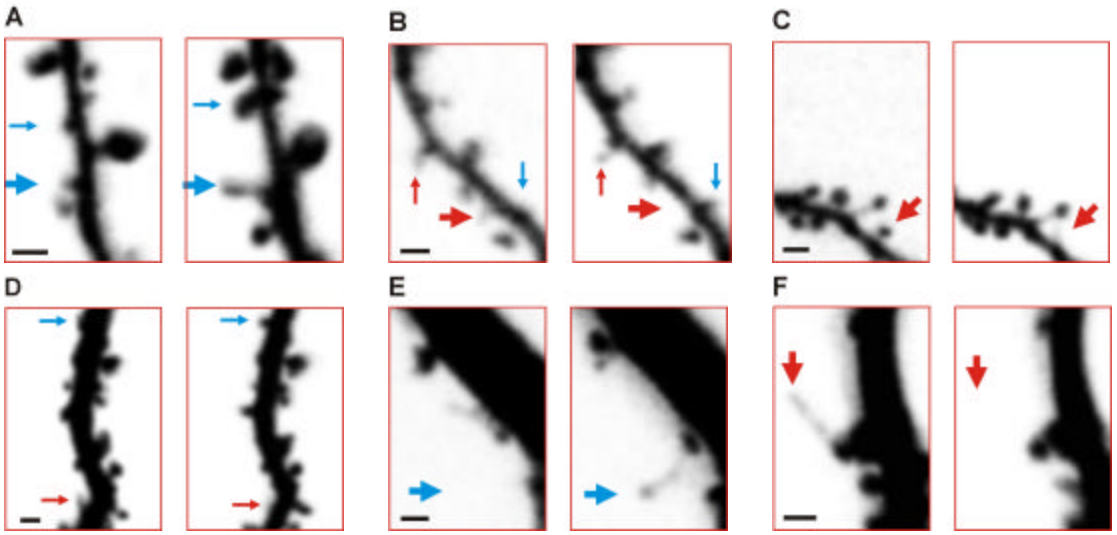

Fig. 17: Examples of 1- and 2-cat changes of spine morphology.
Thin arrows: 1-cat changes; thick arrows: 2-cat change; red arrows: spine retraction; blue arrows: spine growth (scale bars: 1 µm). **A**, Thin arrow: cat 1? 2; thick arrow: cat 1? 3. **B**, Thin red arrow: cat 2? 1; Thick red arrow: cat 2? 0; Thin blue arrow: cat 1? 2. **C**, Red arrow: cat 2? 0. **D**, Blue arrow: 0? cat 1; red arrow: cat 1? 0. **E**, Blue arrow: growth of filopodium. **F**, Red arrow: retraction of filopodium.

Table 3 gives a summary of all the 2-cat changes that were observed 60 min after NMDAR-LTD induction. The control group comprised experiments, in which LFS did not result in depression of synaptic responses to 90% of baseline values or in which the neuron continued to fire action potentials. Comparing the absolute numbers of spine changes between LTD and control experiments, retracting and growing spines appeared to be more prominent in the LTD cases. In the LTD experiments six spines and six filopodia became reduced in size by two categories, whereas only two spines and one filopodium showed a similar behavior in the control experiments. In contrast, two spines

grew in the LTD, and one in the control group. Formation of new filopodia was not observed. For a few experiments the time period of image analysis was extended to 120 min after LTD induction. The number of 2-cat changes at this time point was similar to the results 60 min after LFS.

Considering the large number of analyzed spines (2092 in the LTD group and 1750 in the control group), the number of morphological changes was very small. One has to keep in mind, however, that the number of synapses, which were actually activated by the stimulation, most likely was much smaller than the number of imaged spines. Therefore, a comparison between overall spine number and the number of spine changes might be invalid.

To account for the higher number of LTD experiments (n=14 vs. n=7) and the longer stretches of analyzed dendrites (2720 µm vs. 1228 µm), the mean number of changes per 100 µm dendritic length was calculated: 0.44 shrinking and 0.07 growing spines per 100 µm were observed in the LTD group and 0.24 shrinking and 0.08 growing spines per 100 µm in the control group.

	LTD	Control
Smaller spines	6	2
Filopodia lost	6	1
# of retracting spines/ 100 µm	0.44	0.24
Spines grown	2	1
Filopodia grown	0	0
# of growing spines/ 100 µm.	0.07	0.08
Dendritic length [µm]	2720	1228
S of spines	2092	1750
S of experiments	14	7

Table 3: Summary of 2-cat changes during NMDAR-LTD.

Taken together, these data seem to indicate that NMDAR-LTD promoted the retraction of spines and filopodia. In contrast, NMDAR-LTD did not induce growth of spines or filopodia. However, one has to keep in mind that the number of incidents was rather small, which made it impossible to apply statistical tests. Furthermore, the number of experiments and spines was higher in the LTD group. Therefore, it was difficult to assess

whether the increased numbers of retracting spines and filopodia were really induced by LTD.

If this was indeed the case one would expect that the retraction of spines and filopodia occurred more frequently in NMDAR-LTD experiments than in the controls.

Table 4 and **Table 5** list the distribution of 2-cat spine changes in the NMDAR-LTD and control experiments.

LTD-Experiment	spine density (spines/µm)	Age of the culture (DIV)	smaller spines	larger spines
1	0.59	13	0	0
2	0.67	16	0	0
3	0.74	22	0	0
4	0.75	14	1F	0
5	0.78	15	4F	0
6	0.79	13	0	0
7	0.81	15	3	0
8	0.81	15	0	0
9	0.81	20	0	0
10	0.88	20	1F	0
11	0.93	17	0	0
12	0.95	16	0	0
13	1.01	16	1	1
14	1.10	27	2	1

Table 4: 2-cat spine changes in individual experiments in which LTD was successfully induced (F: Filopodia).

Control-Experiment	spine density (spines/µm)	Age of the culture (DIV)	smaller spines	larger spines
1	0.73	13	0	0
2	0.77	21	0	0
3	0.9	15	0	0
4	0.91	30	0	0
5	1.06	17	1	0
6	1.12	14	1	0
7	1.42	16	1F	1

Table 5: 2-cat spine changes in individual experiments in which no LTD was induced (F: Filopodia).

The experiments 5 and 7 from the LTD group exhibited higher numbers of retracting spines or filopodia. They were of the same age (15 DIV) and had similar spine densities. However, location and distribution of 2-cat changes were different. Whereas in experiment 5 the stimulation electrode was placed at the distal apical dendritic tree (**Fig. 23**; bottom, middle panel), in experiment 7 the electrode was placed basally close to the soma (**Fig. 23**; top, left image). Furthermore, in experiment 5 the changes occurred unclustered on different dendritic branches, whereas in experiment 7 the observed 2-cat changes were strongly clustered.

To determine the probability of spine retraction after LTD, the ratio of experiments, in which spine and filopodium retraction occurred, was calculated. In 43% of the LTD and the control experiments spines retracted indicating that the probability of a neuron to exhibit retracting spines for 2 categories was not increased after NMDAR-LTD induction.

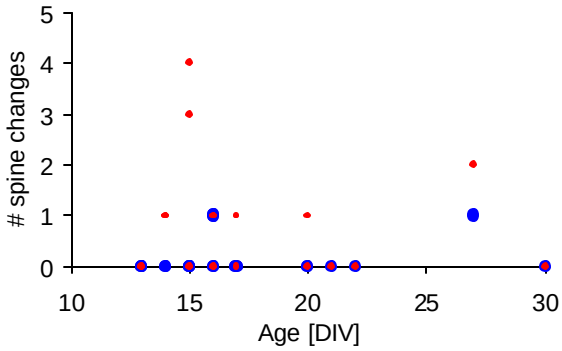

Fig. 18: Relationship between culture age and 2-cat changes.
Red dots: number of retracting spines and filopodia.
Blue dots: growing spines and filopodia.

In addition to LTD, other factors might also influence spine plasticity. Therefore, a possible correlation between age of the culture, spine density, and spine plasticity was analyzed. In **Fig. 18** the numbers of spine changes in a given neuron are plotted against the age of the culture. The relatively large number of spine changes in cultures of 15 DIV (experiments 5 and 7 in **Table 4**) could indicate that 2-cat changes were more likely to occur in young cultures. However, apart from these two neurons, all the others exhibited two, one or no 2-cat changes regardless of their age. Calculation of the linear regression coefficients for growing (R^2=0.04) and retracting (R^2<0.01) spines did not reveal any correlation between age and the number of spine changes.

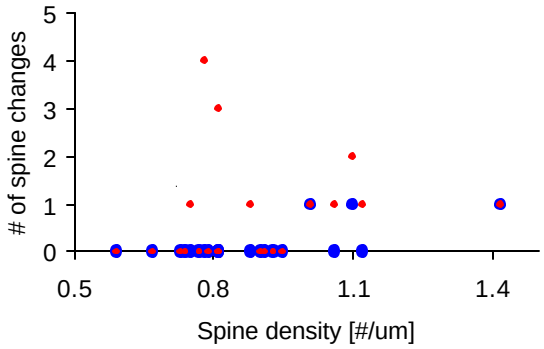

Fig. 19: Relationship between spine density and 2-cat changes.
Red dots: number of retracting spines and filopodia.
Blue dots: growing spines and filopodia.

In a similar manner, the number of 2-cat changes was plotted against the spine density of the respective neuron (**Fig. 19**). Again, there was no relationship between 2-cat changes and spine density. With the exception of two experiments, which exhibited three and four spine changes at comparatively low spine densities, all other experiments exhibited lower numbers of 2-cat changes irrespective of the spine density. This was confirmed by the low values of the regression coefficients for growing (R^2=0.44) and retracting (R^2=0.03) spines.

Thus, spine density and age were unlikely to influence morphological plasticity after LTD.

3.2.2. NMDAR-LTD and 1-category spine changes

To account for the possibility that LTD induced more subtle changes in spine morphology, also 1-cat changes were analyzed. Since spine shape and size were subject to intensity changes in fluorescence and, additionally, grouping of spines into categories was subjective to some degree, subtle changes in the morphology can also be explained by fluctuations that were independent of LTD induction. However, these unspecific fluctuations contributed equally to the LTD and the control group. Therefore, a possible effect of LTD on spine morphology should, nevertheless, be detectable.

Table 6, **Table 7** and **Fig. 20** list a summary of all possible 1-cat changes that were observed during the NMDAR-LTD experiments. As expected, the absolute numbers of 1-cat changes were higher than for 2-cat changes, which allowed application of statistical analysis to reveal significant differences. However, the variation between experiments was large as indicated by the high standard deviation. To assess whether there was a significant difference between the experimental groups at any spine category, growing

(+) and retracting (-) spines of the different categories (1-3) were compared between the two groups. Increased numbers of changing spines were observed for retracting cat 1 spines (1-) and for retracting and growing cat 2 spines (2+, 2-) in the LTD group. A significant difference between the LTD and the control group was revealed for retracting cat 1-spines (small, stubby) ($p = 0.003$; ANOVA). In contrast, the differences between LTD and control experiments were not significant for retracting and growing cat 2 spines ($p_{2-} = 0.125$, $p_{2+} = 0.122$; ANOVA). Furthermore, the ANOVA did not reveal significant differences in numbers of growing or retracting spines of any other category.

LTD-Experiment	0+	1+	1-	2+	2-	3-	Sum
1	6	6	2	3	3	0	**20**
2	1	0	1	2	1	0	**5**
3	8	2	0	0	1	0	**11**
4	1	6	2	4	0	1	**14**
5	0	0	7	1	12	2	**22**
6	0	4	2	1	0	1	**8**
7	0	1	0	2	3	4	**10**
8	12	3	2	5	4	3	**29**
9	0	1	0	0	1	0	**2**
10	1	2	2	2	6	6	**19**
11	0	2	1	1	0	2	**6**
12	4	7	4	3	10	3	**31**
13	0	3	3	0	6	5	**17**
14	0	1	2	1	5	2	**11**
Sum	**33**	**38**	**28**	**25**	**52**	**29**	**205**
Mean #of changes/100 µm	**1.08 (±0.43)**	**1.42 (±0.28)**	**1.02 (±0.23)**	**1.14 (±0.31)**	**2.14 (±0.57)**	**1.41 (±0.57)**	

Table 6: 1-cat spine changes in individual experiments after NMDAR-LTD induction.
Column headings denote spine category and direction of change ('+': growing spines, '-': retracting spines, '0+': *de novo* formation of spines; in brackets: SEM).

Control-Experiment	0+	1+	1-	2+	2-	3-	Sum
1	17	4	0	1	0	0	**22**
2	3	0	0	0	1	1	**5**
3	1	1	1	1	2	3	**9**
4	0	0	0	0	0	0	**0**
5	0	0	0	0	5	1	**6**
6	0	3	0	0	1	2	**6**
7	5	10	0	4	0	2	**21**
Sum	**26**	**18**	**1**	**6**	**9**	**9**	**69**
Mean # of changes/100 µm	**1.89 (±1.07)**	**1.35 (±0.71)**	**0.06 (±0.06)**	**0.41 (±0.28)**	**1.23 (±0.85)**	**0.82 (±0.22)**	

Table 7: 1-cat spine changes in individual experiments without NMDAR-LTD induction.
Column headings denote spine cat and direction of change ('+': growing spines, '-': retracting spines, '0+': *de novo* formation of spines; in brackets: SEM).

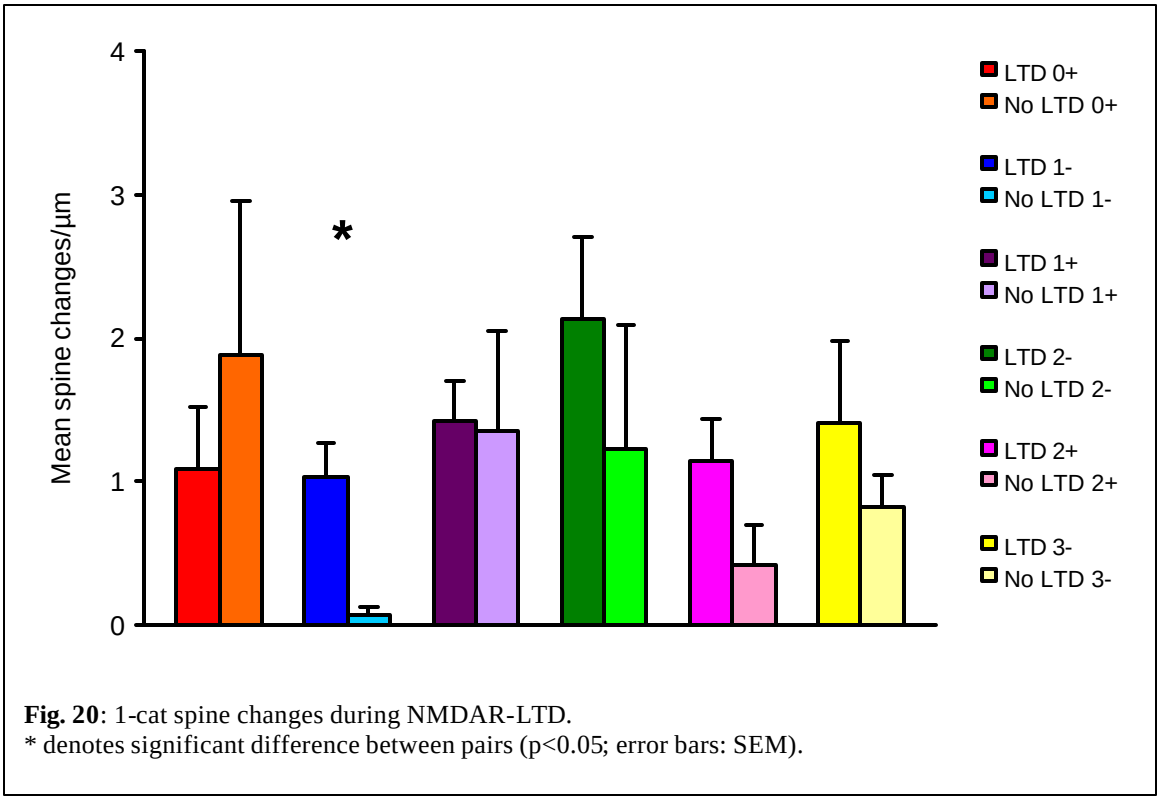

Fig. 20: 1-cat spine changes during NMDAR-LTD.
* denotes significant difference between pairs ($p<0.05$; error bars: SEM).

Comparison of the number of 2-cat changes of the individual experiments with the number of 1-cat changes did not reveal any correlation between the abundance of 2- and 1-cat changes within an experiment (**Table 4, Table 5** and **Table 6**, **Table 7**). Experiments with comparatively high numbers of 2-cat changes (experiments 5, 7, and 14 in the LTD group) exhibited only moderate numbers of 1-cat changes, whereas the experiments with the highest sum of 1-cat changes (experiments 8 and 12 in the LTD group) did not exhibit any 2-cat changes.

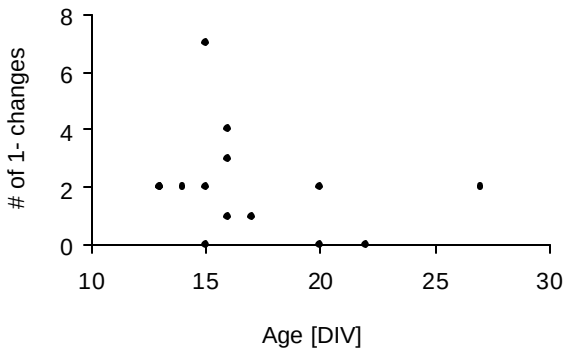

Fig. 21: Relationship between culture age and number of retracting cat 1 spines.

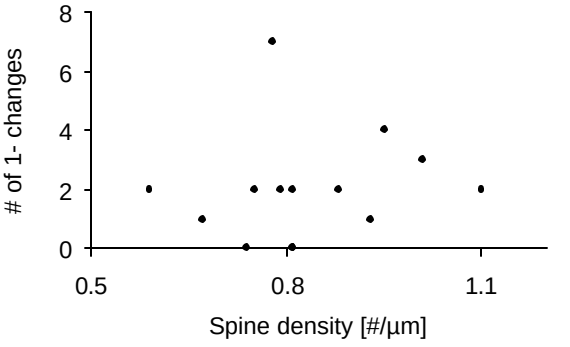

Fig. 22: Relationship between spine density and number of retracting cat 1 spines.

As only the number of retracting cat 1 spines was significantly enhanced by the induction of NMDAR-LTD, this type of morphological change was further analyzed. **Fig. 21** shows the relationship between the number of retracting cat 1 spines and the age of the respective culture for the LTD experiments. The low value of the correlation coefficient (R^2=0.07) suggested that there was no correlation between these parameters.

Additionally, no correlation was found between retracting cat 1 spines and the spine density of the neuron (R^2=0.02;**Fig. 22**).

From this, it can be concluded that NMDAR-LTD significantly increased retracting cat 1 spines independently of age and spine density of the neuron.

3.2.3. Spatial distribution of spine changes

In an earlier study local application of tetanic stimulation has been found to induce formation of filopodia on dendritic branches close to the site of stimulation (Maletic-Savatic et al., 1999). A reasonable explanation for the spatial restriction of the formation of filopodia could be that it resulted in the activation of synapses in close proximity to the stimulation electrode.

In the present study local stimulation was also used to spatially restrict synapse activation and thereby limit potential morphological changes to dendritic branches in the vicinity of the stimulation electrode. Therefore, one would expect morphological changes predominantly to occur around the site of stimulation. To test this assumption, the spatial distribution of morphological changes was analyzed. **Fig. 23** shows images of all the neurons which exhibited 2-cat changes (sites of change are marked with colored dots).

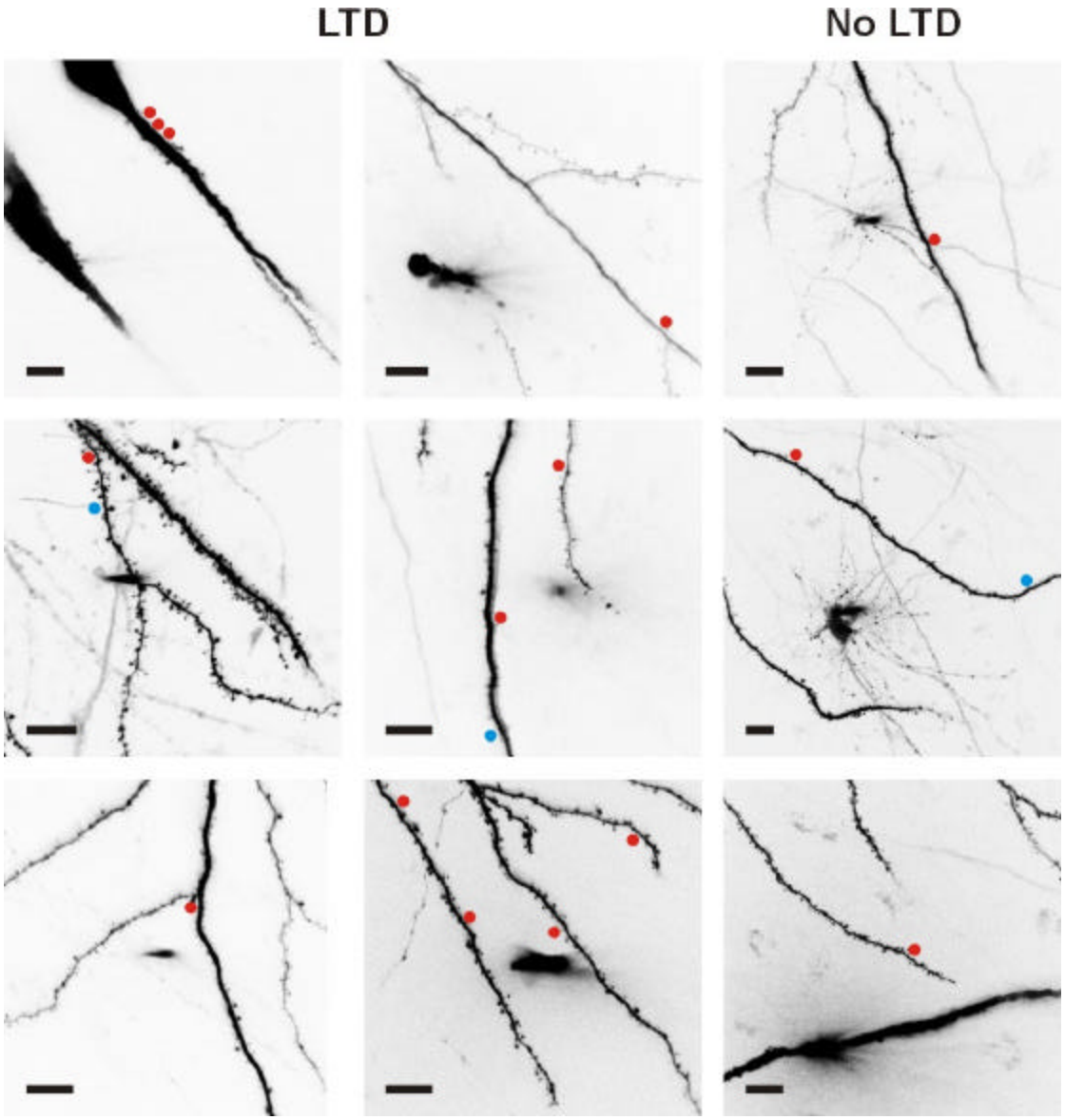

Fig. 23: Spatial distribution of 2-cat changes in individual experiments (scale bars: 10 µm).
Left, middle column: after NMDAR-LTD induction; right column: control condition; red dots: spine retraction; blue dots: spine growth.

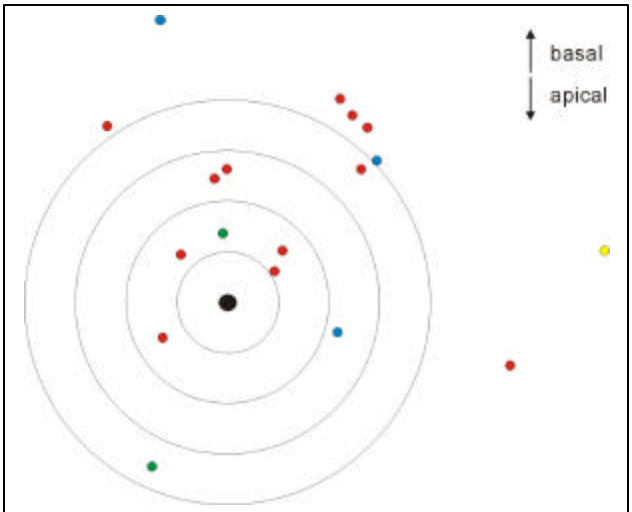

Fig. 24: Superimposed spatial distribution of 2-cat changes (scale bar: 10 µm).
Black dot: Stimulation electrode; red dots: Spine retraction during LTD; Blue dots: Spine retraction without LTD; green dots: Spine growth during LTD; yellow dots: Spine growth without LTD (black rings: 10 µm-increments from the stimulation electrode).

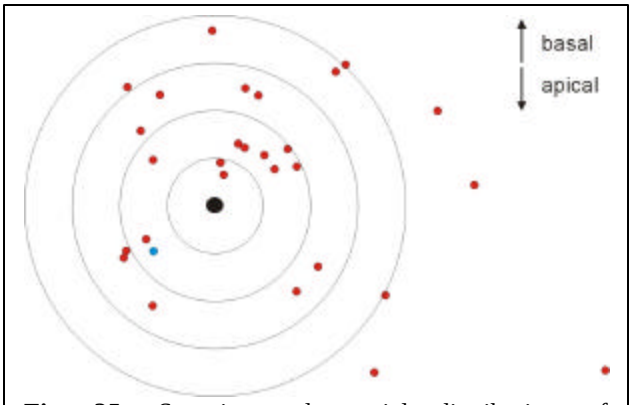

Fig. 25: Superimposed spatial distribution of retracting cat 1 spines (scale bar: 10 µm).
Black dot: Stimulation electrode; red dots: Spine retraction during LTD; Blue dot: Spine retraction without LTD; (black rings: 10 µm-increments from the stimulation electrode).

To analyze the spatial distribution of the sites of spine changes across all experiments, the individual images were size- and orientation-matched and then overlaid, such that the tips of the stimulation electrodes were superimposed.

In **Fig. 24** and **Fig. 25** the incidents of spine changes from the superimposed images were marked and depicted without the respective neurons. **Fig. 24** illustrates the distribution of 2-cat spine changes (colored dots) in relation to the stimulation electrode (black dot in the center). The longest distance between the site of stimulation and an observed 2-cat change was 76 µm. There was no obvious clustering of spine changes around the site of stimulation, but changes appeared to occur predominantly basal to the stimulation electrode (above the site of stimulation, **Fig. 24**). However, this observation could be explained by a higher number of dendritic branches basal to the stimulation electrode (**Fig. 23**; bottom, middle and right image).

Additionally, the spatial distribution of retracting cat 1 spines in the LTD experiments was analyzed. In **Fig. 25** the sites of changes in all experiments were superimposed with respect to the stimulation electrode. The longest distance between the site of stimulation and an observed retracting cat 1 spine was 51 µm. Again, apart from a slight clustering of

changes at the more basal part of the dendritic tree, no obvious clustering around the site of stimulation could be detected.

Given the fact that there is no clear spatial correlation between the sites of 2- and 1-cat spine changes within the area of imaging and the site of stimulation, it could be argued that these changes were not induced by the stimulation. This line of reasoning is weakened, however, by the results of a preliminary study demonstrating that local stimulation activated synapses locally as well as distant to the site of stimulation (up to ~150 µm; U.V. Nägerl, personal communication). Consequently, strong clustering of morphological changes around the site of stimulation is not to be expected. Nevertheless, the study demonstrated that local activation of synapses did occur. Consequently, potential morphological changes should occur in vicinity to the stimulation electrode still holds.

3.2.4. Protein synthesis dependence of NMDAR-LTD

According to the analysis of spine plasticity, 2- and 1-cat morphological changes were rare events. Therefore, it was reasonable to assume that the molecular components that mediated morphological plasticity might not be present throughout the neuron permanently. Rather, they could be synthesized specifically when particular synapses were potentiated or depressed. If this was true synthesis of new proteins should play a role during morphological plasticity.

To test this prediction, the effect of the translation inhibitors anisomycin and cycloheximide on LTD in organotypic cultures was studied. Extracellular recordings of fEPSPs in the pyramidal layer of CA1 were performed, while stimulating Schaffer collaterals in CA1 (**Fig. 26**). Anisomycin or cycloheximide (25 µM) were bath-applied ~1h before LFS and remained present throughout the experiment. As the two inhibitors were dissolved in 0.1% DMSO, application of the same concentration of DMSO served as control condition.

To quantify the amount of LTD for the three groups, the mean fEPSP amplitude 55-60 min after LFS was calculated. Like all other experiments in this study, data were collected and analyzed in a strictly blind fashion (see Methods). Application of LFS

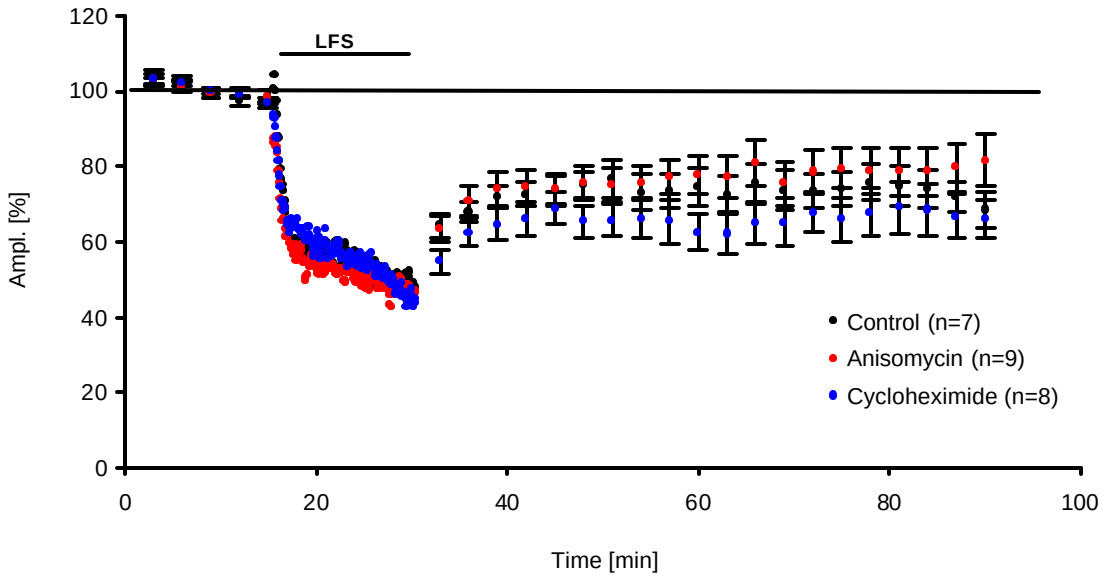

Fig. 26: NMDAR-LTD does not depend on protein synthesis (error bars: SEM).
Bath application of anisomycin or cycloheximide did not change the level of synaptic depression significantly.

resulted in a mean depression of fEPSPs to 80.2 ± 6.8% of the baseline average in the presence of anisomycin (n=9), 65.9 ± 14.4% in the presence of cycloheximide (n=8) and 69.2 ± 11.8% in the presence of DMSO (control; n=7). Statistical comparison of the three groups did not reveal a significant difference ($p = 0.19$; ANOVA). Therefore, NMDAR-LTD did not depend on protein synthesis in organotypic hippocampal cultures 60 min after induction.

A possible objection to this result is that anisomycin and cycloheximide could not exert their effect. In particular, the plasma clot that covered the slice to attach it to the cover slip (see Methods) could act as a mechanical barrier and thereby prevent the diffusion of the inhibitors into the culture.

To test whether anisomycin could penetrate into Gähwiler organotypic cultures, cultures were transfected with an eGFP-expressing adenovirus (provided by A. Gärtner, see Gärtner et al., 2000) and incubated with or without anisomycin overnight. **Fig. 27** illustrates that neurons from transfected cultures expressed eGFP (A, B), whereas eGFP-

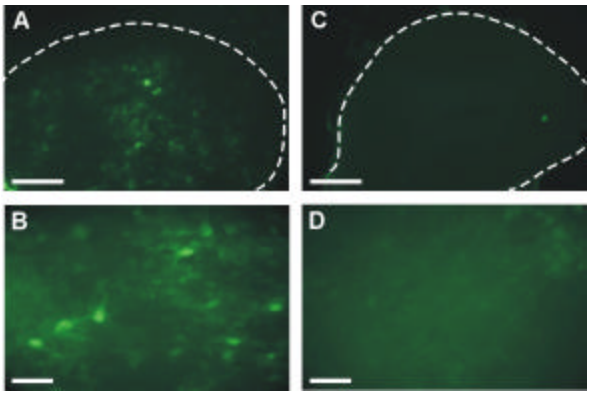

Fig. 27: Fluorescence images of organotypic cultures expressing eGFP. **A**, **B**: Culture was cultivated in normal culture medium. **C**, **D**: Culture was cultivated in anisomycin (25 µM) overnight (**A**, **C**: 5x magnification, scale bar: 400 µm; **B**, **D**: 40x magnification of the CA1 region, scale bar: 40 µm).

expression was abolished in the presence of anisomycin (C, D). Thus, anisomycin could diffuse into Gähwiler cultures and block protein synthesis.

It is important to note, however, that the duration of anisomycin application was much longer than for the extracellular recordings. Thus, it is still possible that anisomycin could not penetrate into the culture during the short incubation period of 60 min.

Taken together, it can be concluded that NMDAR-LTD induced the retraction of small, stubby spines but did not cause formation or retraction of large spines (cat 2, 3) or filopodia within 60 min after LTD induction. Furthermore, NMDAR-LTD did not depend on protein synthesis within this time window.

It has been reported that another form of LTD in the hippocampus, which relies on activation of mGluRs (mGluR-LTD), can be induced chemically by bath-applying mGluR agonists (Stanton et al., 1991). This treatment is likely to cause synaptic depression in a large number of synapses throughout the dendritic tree. If this form of synaptic depression resulted in morphological plasticity of spines, the large number of affected spines should facilitate their detection among the large overall number of dendritic spines. In addition, mGluR-LTD has been reported to be protein synthesis dependent (Weiler and Greenough, 1993; Huber et al., 2000)

For these reasons it was investigated whether mGluR-LTD induced morphological plasticity in dendritic spines.

3.3. mGluR-LTD

Before studying the effect of mGluR-LTD on spine morphology, the optimal parameters for mGluR-LTD induction in Gähwiler organotypic cultures were tested. Extracellular recordings from the pyramidal layer in CA1 revealed that bath application of 50-100 µM of DHPG for 5-10 min resulted in a reliable induction of long-lasting mGluR-LTD in Gähwiler cultures. This was consistent with results obtained from acute hippocampal slices in which 50-100 µM of DHPG was applied for similar periods of time (Palmer et al., 1997).

Fig. 28 shows an example of an extracellular recording of mGluR-LTD. Schaffer collaterals were stimulated in CA1 and fEPSPs were recorded extracellularly in the pyramidal layer in CA1. Application of 100 µM of the mGluR agonist DHPG for 10 min resulted in a drastic reduction of synaptic responses which slowly recovered after agonist-washout but remained well below baseline responses. Applying the 90%-criterion the induction probability of mGluR-LTD was 100%.

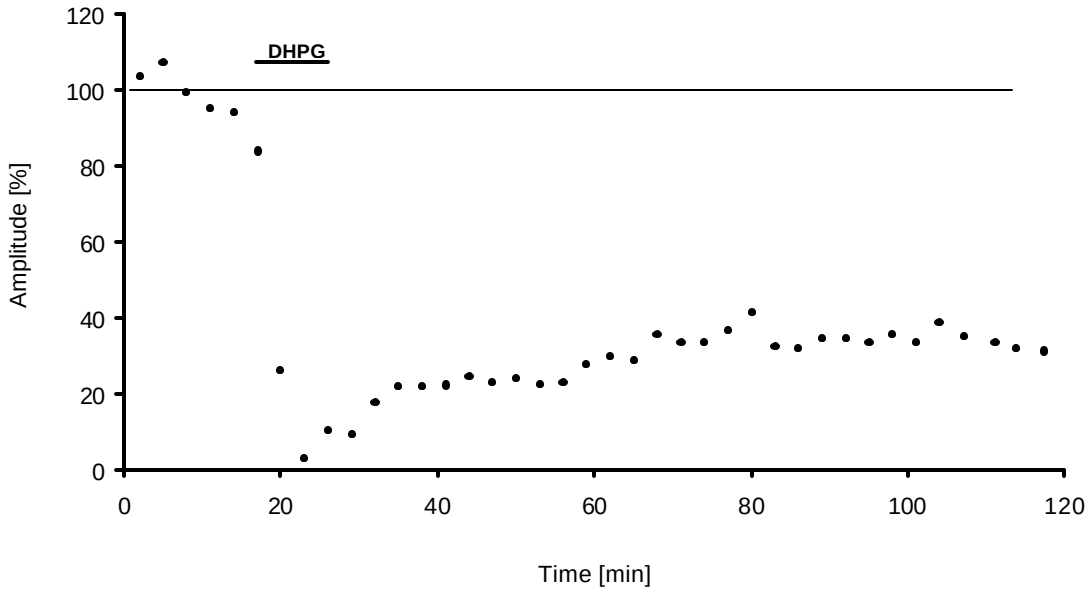

Fig. 28: Example of mGluR-LTD after DHPG-application (100 µM) for 10 min. Application of DHPG resulted in a pronounced depression of EPSP amplitude.

However, in some experiments synaptic responses were abolished completely after DHPG-application. To exclude possible detrimental effects of overdoses of DHPG, the concentration of DHPG was reduced to 50 µM for the subsequent experiments.

In **Fig. 29** Schaffer collaterals were stimulated in CA1 and recordings were done extracellularly in the pyramidal cell layer in CA1. NMDAR-LTD was induced by applying three trains of LFS in quick succession. Whereas the first LFS resulted in strong depression, the second stimulus train yielded only a small further depression. The third LFS did not reduce synaptic responses any further indicating saturation of NMDAR-LTD. In contrast, inducing mGluR-LTD by subsequent application of DHPG caused further synaptic depression illustrating that synapses can be further depressed by mGluR-LTD even if NMDAR-LTD is saturated. Therefore, NMDAR- did not exclude mGluR-LTD in Gähwiler cultures. This confirmed the results of a previous study which demonstrated that NMDAR-LTD did not exclude the subsequent induction of NMDAR-independent LTD (Kemp et al., 2000).

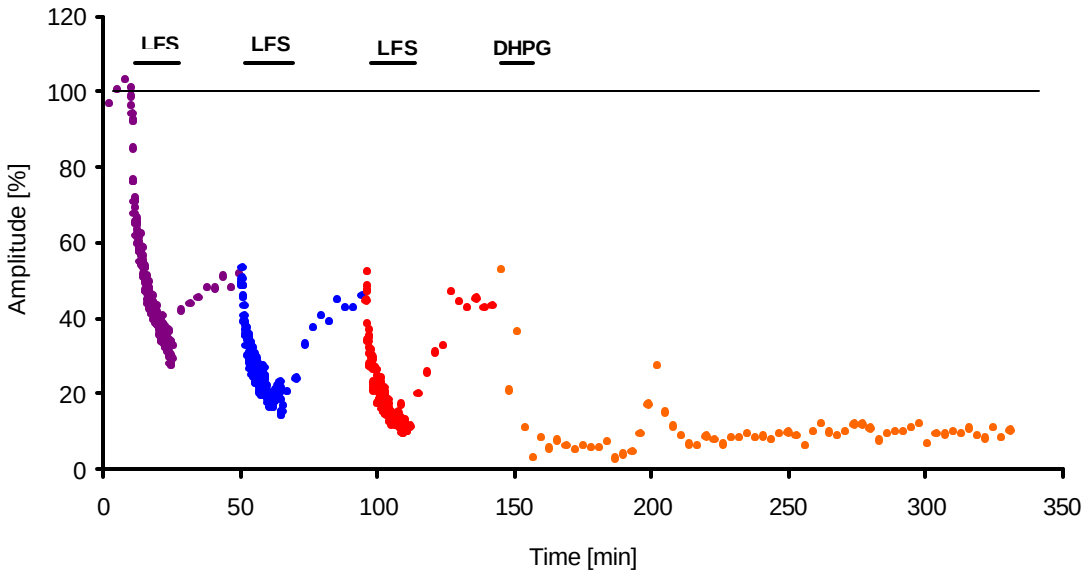

Fig. 29: Example of mGluR and NMDAR-LTD induction in the same culture.
LFS was applied 3x to saturate NMDAR-LTD, followed by DHPG application (50 µM) for 10 min.

To study the effects of mGluR-LTD on spine morphology, intracellular recordings from CA1 pyramidal neurons were performed. EPSPs were evoked by locally stimulating Schaffer collaterals in CA1. Application of 50 µM DHPG for 10 min resulted in a strong depolarization of the cells (~-40 mV) and reduction in synaptic responses (**Fig. 30**). After washout of the agonist the cells slowly recovered from depolarization but often remained slightly depolarized with respect to the membrane potential before DHPG application. DHPG caused a strong reduction in EPSP amplitude. DHPG was dissolved in equimolar concentrations of NaOH in Tyrode solution (50 µM). Application of 50 µM NaOH alone (vehicle solution) for 10 min did not change the pH of the recording Tyrode and served as control condition for the analysis of morphological changes after mGluR-LTD induction.

To analyze morphological changes, images taken before and 60 min after DHPG-application were compared. Since the agonist presumably affected all synapses no correlation between stimulation electrode and depressed synapses was to be expected. For this reason, spines all over the apical dendrites were included into the analysis. Basal dendrites were excluded from the analysis to restrict the numbers of spines to be analyzed.

Fig. 30 and **Fig. 31** illustrate examples of a mGluR-LTD and a control experiment. **Fig. 30A** shows an image of the morphology of a neuron before DHPG application. The magnified images of two arbitrarily chosen regions in **Fig. 30B** illustrate morphological changes of spines. Comparison of the images taken before and after DHPG application revealed one 2-cat change of spine morphology (bottom images). **Fig. 30C** shows that DHPG led to a reduction of EPSP amplitude to 88.0% (average value 50-60 min after DHPG application) of the baseline value. During DHPG application the membrane potential of the neuron became very unstable and the neuron strongly depolarized. The membrane potential was -68.5 mV and slowly recovered to -65.5 mV at 60 min after mGluR-LTD induction (**Fig. 30D**).

In the control experiment the neuron produced EPSPs and action potentials during baseline stimulation. Morphological changes could not be observed during the time course of the experiment (**Fig. 31A**, **B**). Comparison of the images taken at different time points again demonstrates one of the problems of image analysis. In this case, the fluorescence signal of the neuron became more intense in the course of the experiment

leading to an apparent increase in spine size. Application of the vehicle solution did not have any effect on EPSP amplitude (115.0% of the baseline), spiking behavior (**Fig. 31C**), or membrane potential (**Fig. 31D**).

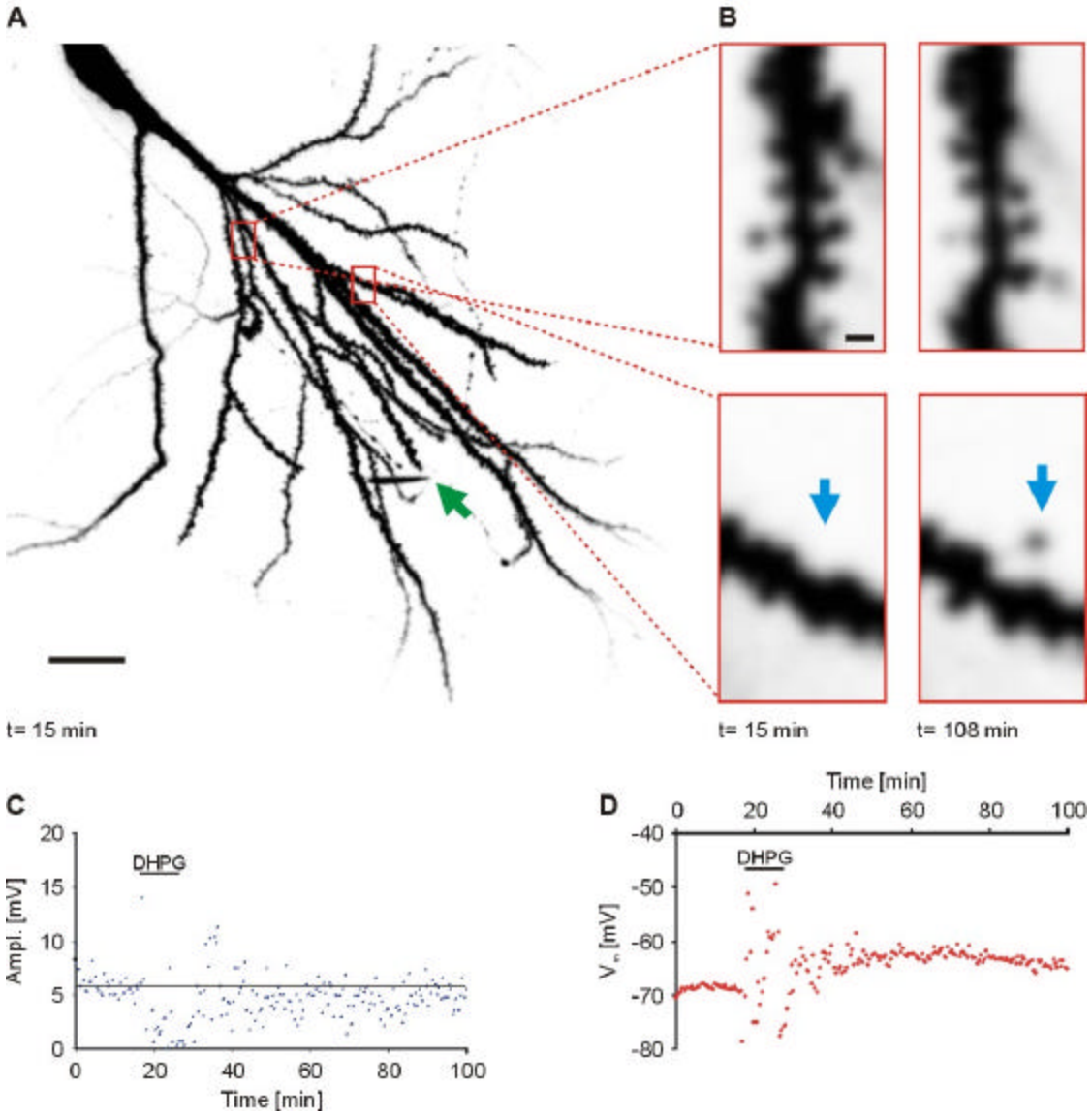

Fig. 30: Example of a mGluR-LTD experiment.
A, Overview image taken before the application of LFS (zoom: 1.0, scale bar: 20 µm). Green arrow: tip of the stimulation electrode. **B,** Magnified images of two different regions of the dendritic tree imaged at the time points indicated (scale bar: 1 µm). Thick blue arrow: spine growth (two categories). **C**, Time course of EPSPs before and after DHPG application. **D**, Time course of membrane potential.

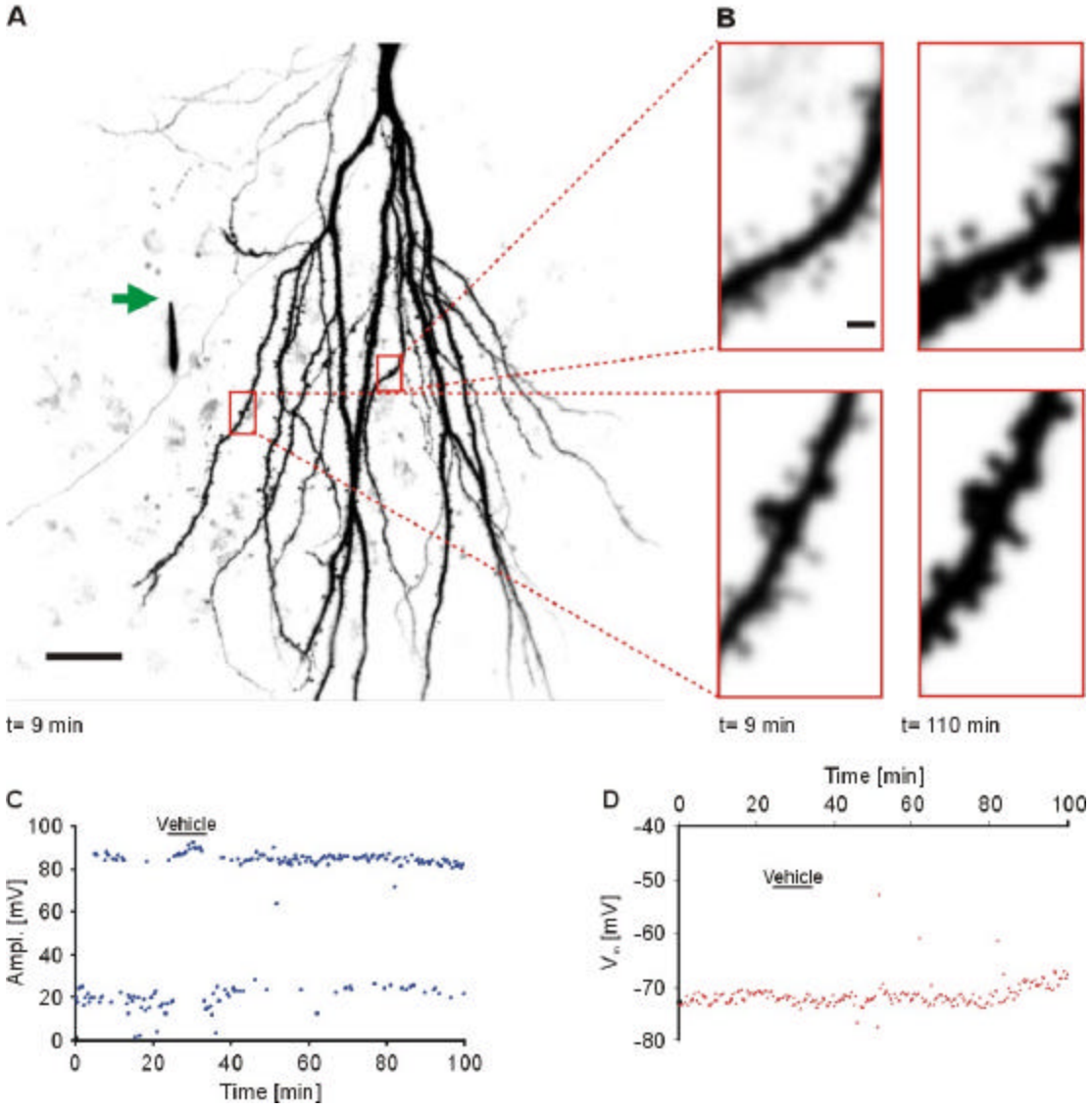

Fig. 31: Example of a mGluR-LTD control experiment.
A, Overview image taken before the application of LFS (zoom: 1.0, scale bar: 20 µm). Green arrow: tip of the stimulation electrode. **B,** Magnified images of two different regions of the dendritic tree imaged at the time points indicated (scale bar: 1 µm). **C**, Time course of EPSPs before and after application of vehicle solution. **D**, Time course of membrane potential.

Applying the 90%-criterion for successful LTD induction, the probability of inducing mGluR-LTD by application of DHPG was 100% (n=7). In the LTD-group the average amount of depression 50-60 min after LTD induction was 32.7% (±13.3%) of the baseline EPSP amplitude. As indicated by the large standard error, there was considerable variation in the amount of depression between experiments after DHPG application.

In the control group (n=4), application of the vehicle solution resulted in an EPSP amplitude of 92.5% (±12.9%). For unknown reasons EPSP amplitudes varied and sometimes decreased during the time course of an experiment. However, not in a single case did application of the vehicle solution result in an immediate change in EPSP amplitude or membrane potential.

3.3.1. mGluR-LTD and 2-category spine changes

Table 8 gives a summary of the changes in spine morphology observed after mGluR-LTD induction or control conditions. Both absolute as well as average numbers of spine changes indicated that more spines changed their morphology after successful mGluR-LTD induction. However, the numbers of morphological changes were very small in relation to the total number of spines analyzed. Given that 2277 spines were analyzed in the mGluR-LTD group and 1918 in the control group only 0.31% of the spines exhibited a reduction in size after LTD-induction (0.052% in the control group) and 0.22% spines increased in size (0.052% in the control group).

	LTD	No LTD
Smaller spines	4	1
Filopodia lost	3	0
Mean # of retraction/exp.	1.17 (±0.54)	0.25 (±0.25)
Spines grown	3	1
Filopodia grown	2	0
Mean # of growth/exp.	0.83 (±0.31)	0.25 (±0.25)
S of spines	2277	1918
S of experiments	6	4

Table 8: Summary of 2-cat spine changes during mGluR-LTD.

To illustrate the numbers of 2-cat changes per experiments, **Table 9** and **Table 10** show the numbers of 2-cat spine changes for the individual experiments:

LTD-Experiment	spine number	smaller spines	larger spines
1	248	2+1F	1F
2	376	0	0
3	440	2F	1F
4	279	0	0
5	531	2	1
6	403	0	2

Table 9: 2-cat spine changes in individual experiments after mGluR-LTD induction (F: filopodia).

Control-Experiment	spine number	smaller spines	larger spines
1	432	1	0
2	746	0	0
3	486	0	0
4	254	0	1

Table 10: 2-cat spine changes in individual experiments without mGluR-LTD induction.

The numbers of morphological changes for the single experiments show that in both groups some experiments did not exhibit 2-cat changes (two out of six in the mGluR-LTD group and two out of four in the control group). Therefore, expression of mGluR-LTD was possible without changes in spine morphology. Together with the low number of spine changes in relation to the total number of spines, these data suggested that mGluR-LTD did not induce 2-cat changes in these neurons.

3.3.2. mGluR-LTD and 1-category spine changes

In addition, 1-cat changes were analyzed to account for the possibility that mGluR-LTD induces more subtle changes in spine morphology. **Table 11** and **Table 12** give a summary of all possible 1-cat changes for the different experiments.

LTD-Experiment	0+	1+	1-	2+	2-	3-
1	1	0	0	0	1	2
2	0	0	0	0	0	0
3	1	0	0	0	0	2
4	0	0	0	0	0	0
5	0	1	1	3	0	2
6	1	1	0	1	0	0
Sum	**3**	**2**	**1**	**4**	**1**	**6**
Average	**0.50 (±0.22)**	**0.33 (±0.21)**	**0.17 (±0.17)**	**0.67 (±0.49)**	**0.17 (±0.17)**	**1.00 (±0.45)**

Table 11: 1-cat spine changes in individual experiments after mGluR-LTD induction.
Column headings denote spine cat and direction of change ('+': growing spines, '-': retracting spines, '0+': *de novo* formation of spines; in brackets: SEM).

Control-Experiment	0+	1+	1-	2+	2-	3-
1	0	0	0	0	1	2
2	0	0	0	0	0	0
3	0	0	0	0	0	2
4	0	1	0	2	0	0
Sum	**0**	**1**	**0**	**2**	**1**	**4**
Average	**0.00**	**0.25 (±0.25)**	**0.00**	**0.50 (±0.50)**	**0.25 (±0.25)**	**1.00 (±0.58)**

Table 12: 1-cat spine changes in individual experiments without mGluR-LTD induction.
Column headings denote spine cat and direction of change ('+' growing spines, '-': retracting spines, '0+': *de novo* formation of spines; in brackets: SEM).

The numbers of 1-cat changes were small and did not allow statistical analysis. No obvious differences between the groups could be observed suggesting that mGluR-LTD did not enhance the plasticity of spine morphology.

Comparison of the absolute numbers of 1-cat changes in the control group for the mGluR-LTD experiments with the numbers of NMDAR-LTD control experiments revealed lower numbers for the mGluR-LTD control experiments. Although this could be

interpreted such that LFS in itself induced 1-cat spine changes, it is more likely that differences in analysis accounted for this discrepancy.

The morphological analysis was performed by an observer who did not know whether a LTD or a control experiment was analyzed. Spines were grouped into 4 categories according to their size (see Methods) and changes in category between images of different time points were analyzed. This categorization of spines was done according to rather subjective criteria which might change with time. To exclude that shifting criteria for spine categorization would mask potential differences between the groups, LTD and their respective control experiments were analyzed in random order. Consequently, shifting criteria would affect both groups equally. However, the subset of mGluR-LTD experiments was analyzed several months after the NMDAR-LTD experiments. Thus, the smaller number of 1-cat changes in the mGluR-LTD control experiments could be explained by a shift in the criteria for spine categorization.

This was further confirmed by presenting the observer arbitrarily chosen examples of NMDAR-LTD experiments which had been analyzed several months before. The second analysis indeed revealed a much smaller number of two- and one-cat changes than the first analysis.

Therefore, differences between the results of the NMDAR- and mGluR-LTD experiments were probably due to shifts in the analysis and were not caused by the different LTD paradigms. The analysis of LTD and the respective control experiments within a subset of experiments was unlikely to be affected.

3.3.3. Protein synthesis dependence of mGluR-LTD

To analyze the role of protein synthesis during mGluR-LTD in organotypic cultures, a series of experiments was conducted in which anisomycin was bath-applied. Extracellular recordings of fEPSPs in the pyramidal layer of CA1 were performed while stimulating Schaffer collaterals in area CA1 (**Fig. 32**). Anisomycin (25 µM) was applied ~1h before LFS application and was present throughout the experiment. Application of 0.1% DMSO served as control condition. There was considerable variation of synaptic responses after DHPG application between experiments. In some experiments synaptic responses were abolished completely, in others they slowly recovered close to baseline levels.

To quantify the amount of depression, the mean fEPSP-amplitude 55-60 min after DHPG-washout was calculated. Average response size was reduced to 64.7 ± 10.3% of baseline in the presence of anisomycin (n = 7) and 50.9 ± 7.5% und control conditions (n = 9). There was no significant difference between the two conditions (p = 0.65; Kolmogorov-Smirnov) suggesting that mGluR-LTD did not depend on protein synthesis in Gähwiler-type organotypic cultures. This result was inconsistent with earlier reports reporting protein synthesis dependence in acute hippocampal slices (Weiler and Greenough, 1993; Huber et al., 2000). A possible explanation for this discrepancy is that mGuR-LTD in acute slice and Gähwiler cultures might differ in its requirement for protein synthesis.

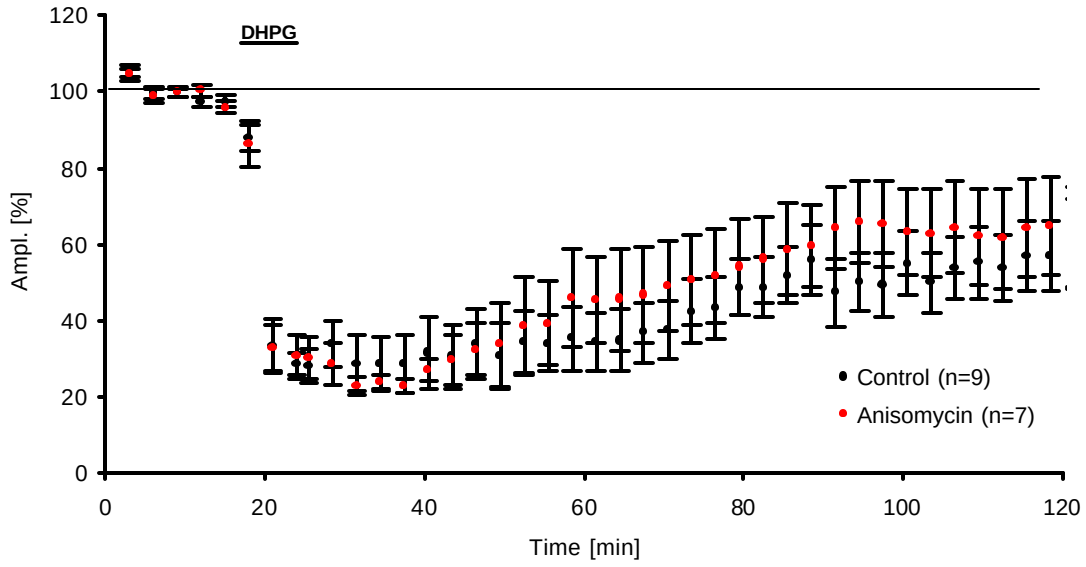

Fig. 32: mGluR-LTD does not depend on protein synthesis (error bars: SEM).

4. Discussion

Synaptic plasticity and morphological changes of dendritic spines have been proposed to be involved in learning and memory. However, the relationship between synaptic and morphological plasticity remained unclear. To address this issue, the present study investigated the effect of LTD on spine morphology in the hippocampus. Local dendritic stimulation and mGluR agonist application was used to induce NMDAR- and mGluR-LTD, respectively. Intracellular recordings from pyramidal neurons and TPLM allowed for simultaneous recordings of synaptic responses and imaging of spine morphology.

Induction of NMDAR-LTD was only observed to enhance the retraction of small spines (cat 1) within 60 min after LTD induction. The numbers of morphological changes of other spine categories were either very low or the differences were not statistically significant. In addition, NMDAR-LTD was independent of protein synthesis, as it was found to be unaffected by protein synthesis inhibitors.

Induction of mGluR-LTD did not result in an increase of morphological plasticity within 60 min following LTD induction. The number of spines exhibiting morphological changes after mGluR-LTD induction was very small relative to the overall number of spines. Also mGluR-LTD was not found to depend on protein synthesis.

4.1. Organotypic slice cultures

In many neurobiological studies, slices of brain tissue are the preparation of choice. They allow electrophysiological studies on brain structures, which are situated deep within the brain and would be difficult to access otherwise. Furthermore, they show preserved cytoarchitecture and connectivity within the slice.

They are not ideally suited for imaging studies, however, as they are several hundred µm thick and the superficial layers contain mainly cellular debris and severed processes resulting from the preparation procedure. Thus, undamaged neurons can only be found towards the central layers of the slice, which makes imaging and pharmacological accessibility more difficult.

In comparison, organotypic cultures combine the advantage of preserved connectivity of brain slices and the transparency of dissociated neuronal cultures. Organotypic cultures are brain slices, which are cultivated under appropriate temperature and medium conditions for weeks and even months (Gähwiler, 1981; Stoppini et al., 1991). During this cultivation period the cellular debris on the surface of the slice is degraded and neuronal processes regenerate. Consequently, undamaged axons and dendrites reach up close to the surface of the culture and, therefore, are easily accessible. In addition, the culture flattens considerably. After two weeks of cultivation the cultures are only one or two cell layers thick and the neurons develop dendritic trees, which are more or less two-dimensional within the plane of the cover slip. This makes organotypic hippocampal cultures an ideal preparation for imaging studies.

Several studies on Gähwiler organotypic slice cultures of the hippocampus could demonstrate that the connectivity and morphological properties of pyramidal neurons resemble those found in acute brain slices or *in situ* (Gähwiler, 1981; Zimmer and Gähwiler, 1984; Frotscher and Gähwiler, 1988). Furthermore, several parameters of synaptic transmission between neurons in area CA3 and CA1, including rise and decay time, duration and latency of EPSPs, have been found to be similar in Gähwiler cultures and acute hippocampal slices (Debanne et al., 1995). In this study paired recordings from single CA3 and CA1 neurons revealed that EPSPs, however, were reported to be 10 times larger than in acute slices, which probably reflects the regeneration of synaptic

connections between CA3 and CA1 following the severing of axons during culture preparation (Debanne et al., 1995). As organotypic cultures are prepared from rat pups at an age at which pyramidal cell axodendrititc excitatory synapses have not yet fully developed (Pokorny and Yamamoto, 1981), the majority of CA3-CA1 connections are formed during the cultivation period. These properties make organotypic hippocampal cultures a good model system to study synaptic transmission and synaptogenesis in the hippocampus.

NMDAR-dependent LTP and LTD have been induced in Gähwiler organotypic cultures using different stimulation paradigms. Pairing presynaptic stimulation of Schaffer collaterals in CA3 with delivery of a postsynaptic current injection into a CA1 pyramidal neuron led to NMDAR-LTP or LTD depending on the order of the stimuli: NMDAR-LTD was induced if the postsynaptic depolarization preceded the presynaptic stimulus (Debanne et al., 1994), whereas reversing the order induced LTP (Debanne et al., 1998). These findings were consistent with the spike timing rule for synaptic plasticity in the hippocampus, as demonstrated in dissociated hippocampal neurons (Bi and Poo, 1998).
In another type of organotypic slice cultures, which is prepared following a slightly different preparation protocol (Müller cultures) (Stoppini et al., 1991), NMDAR-LTD could be induced with the same LFS protocol, which was also used in the present study (Kauderer and Kandel, 2000). The importance of age of the culture was demonstrated by the observation in Müller cultures that the amplitude of EPSPs and the level of LTP is steadily increasing from 20% in one-week old to 70% in four-week old cultures (Collin et al., 1997).
Finally, the morphology of dendrites and spines in organotypic hippocampal cultures also resembles what has been found *in vivo*. The dendritic organization of pyramidal neurons in organotypic cultures consist of a single apical and several basal dendrites emerging from a pyramidal-like cell body (Frotscher and Gähwiler, 1988). Also, the same spine morphologies have been found *in vivo* (Lendvai et al., 2000) and in organotypic cultures (McKinney et al., 1999). Both *in vivo* as well as in organotypic cultures filopodia are particularly prominent during early stages of development and are subsequently replaced by mature spines (Collin et al., 1997; Grutzendler et al., 2002). However, the spine

densities of CA1 neurons in organotypic hippocampal cultures were higher (McKinney et al., 1999) than what was reported *in vivo* (Trachtenberg et al., 2002). This could be explained by excessive sprouting of axons due to lack of input from other brain regions. Consequently, pyramidal neurons from organotypic hippocampal cultures exhibit electrophysiological and morphological properties similar to what has been found *in vivo*, making it an appropriate model system to study the relationship between synaptic and morphological plasticity.

4.2. Image analysis

In order to assess the influence of LTD on spine morphology, spine shape had to be measured reliably. The difficulty to define parameters that would identify spines prevented the automation of spine detection with the help of computer software. Therefore, quantification of morphological changes would have to be performed manually for individual spines. Additionally, to quantify parameters of spine size (e.g. maximal length and width, volume), the physical borders of spines are to be defined. Furthermore, changes in fluorescence intensity between images must be corrected for to avoid changes in spine size due to increases in fluorescence. Preliminary analyses of some experiments measuring spine length did not yield reliable results.

Due to theses difficulties in automation, spine analysis by a human observer yielded the best results in identifying and categorizing spines. Consequently, categorizing spines was done according to subjective criteria rather than on the basis of quantifiable parameters. This subjectivity in categorization could have promoted false positive and false negative results, as also morphological stable spines might be classified into different categories if they lay at the border between two categories. Likewise, changing spines could have been classified into the same category. This was especially true for the analysis of 1-cat changes, as neighboring categories were more difficult to identify. This might explain the higher absolute numbers in comparison with 2-cat changes. However, false positive and negative results should have affected LTD and control groups equally and, therefore, were unlikely to obscure potential differences between the groups. To exclude potential

biases of the analysis, the observer did not know whether the experiment that was analyzed belonged to the LTD or the control group.

Another problem arose by comparing absolute numbers of changes between sets of experiments, which have been analyzed at different time points. Differences in numbers of changes between the sets of experiments could be explained by shifting criteria of spine categorization. The observer might have become more or less stringent over time in counting variations of spine morphology. This could explain why the number of one-cat changes was smaller for the mGluR-LTD than for the NMDAR-LTD experiments. Analysis of the mGluR experiments was performed several months later and meanwhile the observer could have changed the criteria of spine categorization. To test this hypothesis, the same observer reanalyzed some of the NMDAR-LTD experiments and the number of changes compared between both analyses. Indeed, in the second round of analysis fewer changes were counted indicating that the observer had become more stringent in counting changes. Apparently, spines had to deviate from their initial morphology much more to be counted as a true change.

For this reason comparing the number of changes between NMDAR- and mGluR-LTD experiments is misleading and should be considered with care. Analysis of experiments within each NMDAR- and mGluR-LTD series should not be affected by this, however, as LTD and control experiments were analyzed in arbitrary order.

4.3. Morphological changes induced by NMDAR- and mGluR-LTD

4.3.1. Two-category morphological changes

In contrast to recent studies showing that LTP induced formation of spines or filopodia (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999), the results of the present study suggest that synaptic depression do not cause 2-cat changes of spines (**Table 3**) within 60 min after LTD induction. Although absolute numbers of 2-cat changes were higher in the NMDAR-LTD and the mGluR-LTD than in the respective control experiments, the present results do not provide evidence for an increase in spine dynamics after LTD for several reasons.

First, the higher number of morphological changes after successful NMDAR-LTD induction can be explained to some extent by an extraordinary high number of changes in two individual experiments (**Table 4**, **Table 5**). In addition, the numbers of morphological changes in the single experiments were too small for statistical tests to detect significant changes between the NMDAR-LTD and control group (**Table 4**, **Table 5**). Therefore, it could not be verified that the observed differences were statistically significant.

Absolute numbers of growing and retracting spines were higher also for the mGluR-LTD than for the control experiments. However, comparing the number of spine changes with the total number of analyzed spines revealed that only 0.31% of the spines analyzed retracted and 0.22% of all spines exhibited a size increase. Assuming that bath application of the mGluR agonist should cause wide-spread depression in a large number of synapses all over the dendritic tree, the low numbers of plastic spines suggests that induction of mGluR-LTD did not affect spine morphology.

Second, calculating the ratio between the number of experiments, in which morphological changes occurred, and the overall number of experiments revealed that the probability of morphological changes to occur was low and did not differ between the NMDAR-LTD and control group (43%; **Table 3**). Consequently, in the majority of experiments NMDAR-LTD did not induce any 2-cat morphological changes.

Third, comparing the numbers of 2-cat changes in spine morphology after NMDAR-LTD with previous results for NMDAR-LTP indicates that the level of spine changes observed after LTD was considerably lower than what has been found for LTP (**Table 13**). In an earlier study stimulation of Schaffer collaterals and intracellular recordings from CA1 pyramidal neurons was used to induce and record NMDAR-LTP, while spine morphology was imaged with TPLM. Applying a Cd^{2+}/low Ca^{2+} solution to the bath blocked synaptic transmission in the culture (Engert and Bonhoeffer, 1999). With the help of a local superfusion technique synaptic transmission could be unblocked within a small region of the dendritic tree of the recorded neuron. This allowed to compare morphological plasticity in a region, in which LTP most likely occurred (superfusion spot), and a control region with blocked synaptic transmission (off spot).

Table 13 illustrates that after LTP the amount of morphological changes per 100 µm dendritic length was much higher than what was observed for LTD in the present study. During LTP 6.0 spines per 100 µm dendrite exhibited growth of at least two categories. In comparison, the numbers of growing (0.1 spines/100 µm) as well as retracting spines (0.4 spines/100 µm) after NMDAR-LTD induction were much lower. These numbers were rather comparable to the values for retracting spines after LTP within the superfusion spot (0.2 spines/100 µm) and for growing spines outside the spot (0.2 spines/100 µm). Furthermore, lack of synaptic transmission seemed to destabilize spines more than NMDAR-LTD (1.2 spines/100 µm).

Consequently, comparing the results for NMDAR-LTP and –LTD, strongly suggests that the low numbers of morphological plasticity observed in the present study were not specific to the induction of NMDAR-LTD.

	LTD	No LTD	LTP	No LTP	Off spot
Spine density [#/µm]	0.83	0.99	0.40	0.55	0.50
Smaller spines	12	3	1	0	26
smaller spines/ 100 µm	0.4	0.2	0.2	0	1.2
Larger spines	2	1	32	0	4
larger spines/ 100 µm	0.1	0.1	6.0	0	0.2

Table 13: Comparison of morphological changes induced by NMDAR-LTD in the present study and induced by LTP in an earlier study (Engert and Bonhoeffer, 1999).

Fourth, in another recent study NMDAR-LTD was not found to increase the number of 2-cat changes (Polnau, 2003). The experimental approach of this study was the same as for the effects of NMDAR-LTP on spine morphology (Engert and Bonhoeffer, 1999). **Table 14** shows the numbers of 2-cat spine changes under the different experimental conditions. The absolute numbers of retracting and growing spines were very small. Calculating the ratio of plastic spines relative to the over all numbers of analyzed spines revealed that less than 0.5% of the analyzed spines exhibited morphological changes in any condition. In comparison to the results for NMDAR-LTP, these numbers were much lower suggesting that NMDAR-LTD indeed did not cause changes in spine number. Similarly, the numbers for 1-cat changes were also very small and did not show any obvious differences between the LTD and the control conditions.

	LTD		No LTD		APV	Superfusion control (in spot)
	In spot	Off spot	In spot	Off spot		
Total # of spines	1881	2954	502	719	2117	1431
Smaller spines	2	0	1	0	0	1
Larger spines	2	2	0	0	3	0

Table 14: Effects of NMDAR-LTD on spine morphology (Polnau, 2003).
While LTD was exclusively induced within a superfusion spot (in spot) synaptic transmission was blocked in the rest of the slice (off spot). Different control conditions were used: no LTD: LFS did not lead to LTD; APV: LFS in the presence of the NMDAR-blocker APV; superfusion control: No LFS in the superfusion spot.

Taken together, the low numbers of morphological changes in the present study did not suggest that NMDAR- or mGluR-LTD caused 2-cat changes of spine morphology within 60 min after LTD induction.

However, although occasional analysis of longer time periods after LTD induction (up to 120 min) also did not reveal increased morphological plasticity, it cannot be excluded that LTD-induced morphological changes occur only later. Although LTP has been found to induce formation of new spines within 20-30 min after induction, it is conceivable that especially retraction of spines might take longer. Assuming that spine retraction would require prior degradation of synaptic structures before the spine itself can be disassembled, spine retraction might occur with a delayed onset.

The high degree of morphological stability in the present study is also in line with recent results *in vivo*. Spines have been reported to exhibit remarkable stability over days and weeks, in the sense that these spines did not appear or disappear during this period. In the visual cortex of one-month-old mice ~90% of the spines remained stable over three days (Grutzendler et al., 2002). In adult mice the majority of spines (90%) remained stable over a two-month period. Although most spines remained visible for days and weeks, however, changes in morphology did occur.

In contrast, in the mouse barrel cortex of 6-10-week old mice only ~60% of the spines have been found to remain stable for more than eight days (Trachtenberg et al., 2002). 17% of the spines appeared or disappeared within one day.

Consequently, the differences in the numbers of stable spines between the two studies suggest that morphological stability of spines might be different between brain regions. Nevertheless, it can be concluded that in the intact brain *de novo* formation or the total loss of spines are relatively rare events. The majority of spines seem to remain stable over long periods of time.

4.3.2. One-category morphological changes

In contrast to 2-cat changes, analysis of 1-cat changes showed that the numbers of these more subtle changes of spine morphology were statistically different between the NMDAR-LTD and control experiments (**Table 6**).

Comparing the means of all the possible 1-cat changes of NMDAR-LTD experiments and the control group, significantly more small spines (category 1) were observed to retract in the LTD than in the control group (**Fig. 20**). For the other spine categories the differences in number of 1-cat changes were not statistically different between the groups. Therefore, the data suggest that NMDAR-LTD specifically promoted the disappearance of small, stubby spines.

In mGluR-LTD experiments absolute numbers for 1-cat changes were too small to test for statistically significant changes between groups (**Table 11**, **Table 12**). Considering that many synapses potentially were affected by mGluR activation, the low numbers of morphological changes suggest that mGluR-LTD did not increase morphological plasticity.

The result that cat 1 spines were preferentially lost during NMDAR-LTD is remarkable because small spines are likely candidates for morphological plasticity after LTD. Spine size has been found to be correlated with PSD dimensions, the number of postsynaptic receptors and number of presynaptic vesicles (Nusser et al., 1998; Schikorski and Stevens, 1997). Thus, stubby spines contain smaller synapses, which produce smaller

synaptic responses. Therefore, it is reasonable to assume that small spines with reduced synaptic efficacy are especially prone to removal, whereas big spines with more efficient synapses are maintained.

This line of reasoning is further strengthened by the model of 'synaptic competition', which has been put forward to explain synaptic pruning during neuronal development (Barde, 1989). It proposes that synapses compete with each other for limited amounts of a diffusible factor that is crucial for synaptic maintenance (Barde, 1989; Bonhoeffer, 1996). The postsynaptic cell would release this factor upon depolarization. Synchronously active presynaptic sites can take up more of the factor, whereas asynchronously active sites or sites that do not receive any input take up less. It has also been suggested that bigger synapses receive more of the factor making them more likely to be maintained.

This competition for a synaptic 'survival factor' could offer an explanation for the finding that particularly small, stubby spines are lost after NMDAR-LTD. Synapse pruning during LTD might affect smaller synapses rather than larger ones as they are less active and therefore receive less survival factor.

In particular, the members of the neurotrophin family are potential candidates for the above mentioned survival factor and have been implicated in mediating activity-dependent morphological plasticity. Especially brain-derived neurotrophic factor (BDNF) is known to be involved in LTP and LTD. The lack of BDNF, either as a result of gene or protein inactivation, leads to a profound inhibition of NMDAR-LTP (Korte et al., 1995; Patterson et al., 1996). In line with this, the addition of BDNF to hippocampal slices isolated from wildtype animals leads to a long-lasting enhancement of synaptic transmission (Kang and Schuman, 1995). Conversely, application of exogenous BDNF prevents LTD in the visual cortex (Kinoshita et al., 1999). Culturing dissociated hippocampal neurons in BDNF induces formation of synapses on these neurons (Vicario-Abejon et al., 1998). Overexpressing BDNF in slices from the visual cortex was found to lead to destabilization of spines resulting in increased growth and retraction rates (Horch et al., 1999) also indicating that BDNF is important for synapse (and spine) formation and maintenance.

The issue of how spine retraction and growth could be mediated molecularly is largely unclear. A hypothesis has been put forward, suggesting a common mechanism for spine

formation and retraction (Segal et al., 2000). According to this theory, local Ca^{2+}-levels influence the morphology of spines: high Ca^{2+}-concentrations cause rapid shrinkage and collapse of spines, whereas moderate levels lead to spine elongation. Furthermore, it is suggested that a central, somatic rise in Ca^{2+}-concentration will lead to the formation of novel spines or their elimination all over the dendritic tree.

However, this line of reasoning does not fit to another, more widely accepted theory, which states that LTP and LTD are mediated by high and moderate Ca^{2+}-levels, respectively (Lisman, 1989). Consequently, the observation that LTP leads to spine formation and LTD to the elimination of stubby spines would link large increases in Ca^{2+} to spine formation and moderate rises to spine retraction.

4.3.3. Morphological plasticity during NMDAR-LTP

In principle, as spine size and receptor and vesicle number are correlated, larger spines should reflect enhanced synaptic transmission. Therefore, one would expect LTP to induce, if anything, growth of existing spines or even *de novo* formation of spines.

Early reports studying the effect of LTP on spine number and morphology had to rely on statistical analysis of stimulated and unstimulated neurons. Therefore, they were prone to sampling and statistical artifacts and often yielded conflicting results.

LTP-inducing stimulation has been correlated with an increase in spine size (Fifkova and Van Harreveld, 1977), synapse area (Desmond and Levy, 1986), and other parameters of spine geometry (Lee et al., 1980; Buchs and Müller, 1996). Synaptic potentiation in the gyrus dentatus resulted in a 30% increase in spine number without a change in spine dimensions (Trommald et al., 1996). Induction of chemical LTP using tetraethyl-ammonium (TEA) was reported to lead to lengthening and angular displacement of spines (Hosokawa et al., 1995).

The mean spine area has been found to increase in the dentate gyrus following LTP (Van Harreveld and Fifkova, 1975). In contrast, in CA1 the spine area has been reported to remain constant. Instead, the number of shaft synapses increased and the variability of spines decreased (Lee et al., 1980). Desmond and Levy counted higher numbers of a particular subtype of spines (cup-shaped) in the dentate gyrus without a concomitant

increase in overall spine numbers (Desmond and Levy, 1986). In a later study they found that LTP implied a decrease in multiple synaptic contacts and polyribosome associated synapses (Desmond and Levy, 1990). In contrast, LTP in the CA1-region seemed to involve a reduction of cup-shaped spines and an increase in small, stubby spines (Chang and Greenough, 1984).

In a more recent study spine size and number appeared to remain constant after synapse potentiation (Sorra and Harris, 1998). In line with this, rates of spine splitting were not found to change following LTP (Fiala et al., 2002), which has been suggested as a potential mechanism for spine formation. Even reduction in spine density following LTP has been observed in the dentate gyrus (Rusakov et al., 1997). Similarly, 24h-overexpression of BDNF which is known to be required for LTP (Korte et al., 1996) led to reduced density and destabilization of spines in ferret visual cortex slice cultures (Horch et al., 1999).

In these studies comparisons were made between differentially manipulated cultures. Therefore, the conflicting results could easily be explained by high spine number variability between cultures, small fractions of manipulated synapses and varying time windows during which spine modifications were analyzed. In addition, the fact that different culture systems, developmental stages and hippocampal areas were investigated might add further variation to the results.

Recent advances in imaging technology allowed for time-lapse imaging of living neurons. In combination with intracellular recordings the morphology as well as the synaptic responses of a single cell could be traced over time, thereby circumventing sampling errors and the need of statistics.

Using intracellular recordings and TPLM in combination with local superfusion, induction of NMDAR-LTP has been demonstrated to lead to formation of 6.0 spines/ 100 µm within the superfusion spot (Engert and Bonhoeffer, 1999). In comparison, outside the superfusion spot 0.2 spines/100 µm were lost.

Although this might be evidence for a lack of effect of LTD on spine morphology in the present study, one has to keep in mind that both studies used different methodological

approaches. These differences could influence the potential of neurons for morphological plasticity.

First, in the superfusion approach, synaptic transmission was blocked in the whole culture and only within the superfusion spot synaptic transmission was possible. Different levels of synaptic activity have been reported to influence the spine density on dendrites (McKinney et al., 1999; Kirov and Harris, 1999). Therefore, it is conceivable that silencing the culture increased the potential of neurons to exhibit morphological changes, which could explain the higher numbers of spine changes in the LTP-study. Also, applying a high [Ca^{2+}] solution as superfusion medium could alter the potential of spines in the superfusion spot to undergo morphological changes.

Another potentially important difference affects the neurons themselves. In the LTP-study spine densities of the neurons were considerably lower (0.48 spines/µm) than what was found for neurons in the present study (0.88 spines/µm). This could indicate more fundamental differences in the condition of the cultures. Given that blocking spontaneous activity led to a reduction in spine density in organotypic hippocampal cultures (McKinney et al., 1999), the high spine densities in cultures used for the present study might indicate high levels of baseline electrical activity. However, the fact that no correlation between spine density and morphological changes could be observed (**Fig. 19**) argues against spine density to affect LTD-dependent spine plasticity.

NMDAR-LTP has also been associated with growth of filopodia. Using TPLM in combination with local application of tetanic stimulation to induce LTP, a 19% increase in protrusion density close to the site of stimulation was observed (Maletic-Savatic et al., 1999). In contrast to the superfusion study, filopodia were formed instead of spines indicating mechanistic differences of the evoked growth of protrusions between the two LTP studies. A possible explanation for this difference could be that labeling of neurons was achieved via viral transfection with an eGFP-expressing Sindbis-virus. This virus is known to have detrimental effects on the host cell by affecting its protein synthesis machinery (Frolov et al., 1996; Craig, 1998).

Using 2-4 week old organotypic Müller cultures, the spine density of those neurons was found to be 0.52 spines/µm, which was similar to the value of the superfusion study but again lower than the spine densities of the present study.

In this study local tetanic stimulation induced morphological changes within 30 µm around the stimulation electrode. This could be explained by activation of axons that were likely to contact a nearby dendritic branch. To spatially restrict the area where morphological changes were to be expected, local stimulation was also used in the present study. Following this logic, morphological changes would be spatially clustered on dendritic sites in vicinity of the stimulation electrode.

Analysis of the spatial distribution of 1- and 2-cat morphological changes revealed that within the area of imaging the numbers of morphological changes were smaller than in the LTP-study. In addition, the sites of change were not clustered around the stimulation electrode (**Fig. 24, Fig. 25**). Since morphological changes were also observed up to 80 µm away from the stimulation electrode, this might argue that these changes were not induced by the stimulation.

However, preliminary results of a study using Ca^{2+} imaging to visualize synaptic activation by local stimulation demonstrated synaptic activation at local as well as distant sites on the apical and the basal dendritic tree (U.V. Nägerl, personal communication). It is not clear, however, whether the high Ca^{2+}/low Mg^{2+} solution, that was used to evoke Ca^{2+} influx into the dendrite via NMDARs, might change the number of activated synapses. Nevertheless, this result suggests that local stimulation can cause wide spread activation of synapses.

This was consistent with neuroanatomical studies from organotypic cultures (Frotscher and Gähwiler, 1988) showing that axon collaterals of CA3 pyramidal neurons project to the apical and basal dendrites of CA1 neurons. Additionally, *in situ* a high degree of axonal branching was observed in Schaffer collaterals from CA3 pyramidal neurons resulting in a dense meshwork of axonal collaterals criss-crossing within CA1 (Sik et al., 1993).

According to these results, the branching pattern of Schaffer collaterals makes it difficult to predict to which dendritic sites of a CA1 neuron synaptic contacts are formed. Consequently, local application of LFS could have induced NMDAR-LTD at synapses all

over the dendritic tree without spatial clustering of potential morphological changes. Nevertheless, as local stimulation did cause local activation of synapses, potential morphological changes of spines induced by NMDAR-LTD would occur in vicinity of the site of stimulation. The fact that they occurred less often than what was found for LTP indicates that NMDAR-LTD did not induce morphological changes.

4.3.4. Homeostatic plasticity

Given the finding that LTP can cause rapid formation of new spines, the lack of morphological changes following LTD is surprising. Just as a neuron has the capability to strengthen and weaken individual synapses one would expect that synapse and spine number might also be subject to up- as well as downregulation. Consequently, if LTP leads to formation of new spines neurons should have a mechanism for spine removal to keep the overall number constant. Removal of connections, which had undergone LTD, could constitute an efficient mechanism of how neurons specifically maintain highly active and therefore important connections and remove redundant ones.

Alternatively, it is also conceivable that spines and synapses are removed constantly and that only LTP is able to specifically add new connections. This would make LTP a prerequisite for synapses to be maintained. Unpotentiated as well as depressed connections would therefore be removed unspecifically regardless of whether they have undergone LTD or not. This scenario would not require LTD to counterbalance the morphological effects of LTP.

Both LTD-specific and -unspecific loss of spines could serve the principle of homeostatic plasticity, which has been proposed to be an important principle of neuronal adaptation to changing activity patterns (for review, s. Turrigiano, 1999). A substantial body of evidence has accumulated that homeostatic plasticity serves to maintain the level of synaptic input onto a neuron constant within a dynamic range. In contrast to LTP and LTD, which exert their effects rather rapidly and specifically to a subset of synapses, homeostatic plasticity has been found to act at the time scale of hours or days and affect synapses unspecifically. Regulation of neuronal excitability (Desai et al., 1999), synaptic strength (‘synaptic scaling’) (Turrigiano et al., 1998; O'Brien et al., 1998) and of the

induction-threshold for LTD and LTP (‘sliding synaptic modification threshold’, s. Bear, 1995; Mayford et al., 1995; Abraham et al., 2001) have been demonstrated to mediate homeostatic plasticity.

But also changes in synapse number have been implicated in counteracting changes in activity levels. Prolonged universal blockade of synaptic transmission in acute hippocampal slices led to a general increase in spine number after several hours (Kirov and Harris, 1999), which might compensate for the reduced synaptic activity after the slice preparation. In contrast, reducing synaptic activity in the barrel cortex of mice by abolishing sensory input from the whiskers was found to increase the turnover rate of spines rather than spine numbers (Trachtenberg et al., 2002). Therefore, it remains unclear whether different levels of synaptic activity simply induce a compensatory regulation of spine number or whether synapses are destabilized and actively search for new presynaptic partners.

4.4. Age-dependency of morphological plasticity

To assess the stability of spine number and morphology of pyramidal neurons in Gähwiler cultures, individual neurons were imaged at various time points after preparation. Although the morphological changes observed in these experiments were not quantified, a clear difference between younger and older cultures was found. Whereas early after culture preparation neurons were largely devoid of dendritic spines and rather had dendritic protrusions and filopodia, which might represent immature precursors of spines (**Fig. 11**), dendrites of neurons between 2-4 weeks in culture were densely covered with mature spines of various shapes and sizes (**Fig. 13**). Furthermore, the turnover rate in young cultures was high (**Fig. 12**), whereas older cultures exhibited a high degree of morphological stability over time.

These findings confirm previous reports on morphological development in organotypic cultures. It has been shown that immediately after plating the slices on the glass cover slip pyramidal neurons in CA1 appeared to exhibit normal dendritic morphology with only few spines (Gähwiler, 1981). At 1 DIV neurons predominantly bore highly dynamic filopodia-like processes (Dailey and Smith, 1996). Using time-lapse confocal microscopy, this study also showed that subsequently spine-like protrusions appeared (protospines), which were then replaced by mature spines of high stability. These protospines might be related to the dendritic protrusions that were observed at 4 DIV in the present study.

Similar results have been obtained from electron microscopic studies showing that at early developmental stages (P1) most synapses were shaft synapses, whereas with increasing age synapses on filopodia and spines predominated (Fiala et al., 1998). Thus, the density of dendritic protrusion gradually increases with age also *in situ.* Therefore, the morphological changes in organotypic cultures during the first two weeks in culture were unlikely to represent pathological phenomena induced by the culture preparation but might rather reflect developmental processes as they happen during normal development.

Thus, studying the phenomenology and dynamics of synaptogenesis in organotypic cultures can therefore offer valuable insight into these processes like they occur during neuronal development *in vivo.*

4.5. Protein-synthesis dependence of NMDAR- and mGluR-LTD

To assess the role of protein synthesis during LTD-dependent retraction of small spines, the effect of application of translation inhibitors on NMDAR- and mGluR-LTD was studied. Inhibition of protein synthesis did not have an effect on NMDAR- or mGluR-LTD in Gähwiler cultures (**Fig. 26**, **Fig. 32**).

This contradicts previous results, which reported that NMDAR-LTD required protein synthesis in organotypic Müller cultures (Kauderer and Kandel, 2000). A possible explanation for this discrepancy is the difference in the culturing technique between Müller and Gähwiler cultures. Gähwiler-cultures are covered by a plasma clot which attaches the tissue on top of a cover slip. This could impair the accessibility of drugs to the culture. Although anisomycin had the potential to penetrate the plasma clot and affect protein synthesis in the culture (**Fig. 27**), it could not be excluded that the diffusability was slowed down so that the inhibitors could not penetrate into the culture during an experiment. Therefore, longer incubation times might be necessary to reveal a potential protein synthesis dependence of NMDAR-LTD.

However, NMDAR-LTD could not be blocked with protein synthesis inhibitors in acute hippocampal slices (Huber et al., 2000) suggesting that NMDAR-LTD in Müller cultures might differ in their requirement for new proteins from LTD in acute slices. It is conceivable that there might be also a difference in NMDAR-LTD between Gähwiler and Müller cultures.

In acute hippocampal slices mGluR-LTD has been shown to depend on protein synthesis (Weiler and Greenough, 1993; Huber et al., 2000). Again, this discrepancy to the results of the present study can be explained by the fact that different tissue preparations were used. In acute hippocampal slices and in organotypic cultures mGluR-LTD might differ in their requirement of protein synthesis.

In all of the studies, which demonstrated protein synthesis dependence of LTD, protein synthesis inhibition exerted its effect immediately after LTD induction. This suggests that LTD depends on new proteins already during LTD induction or immediately thereafter.

This is in contrast to NMDAR-LTP, which has been reported to be independent of protein synthesis during the first hour after LTP induction. It is now generally accepted

that NMDAR-LTP consists of a protein-synthesis independent early-phase (< 1h after induction) and a late-phase (> 1h after induction) during which proteins synthesis is required (Frey et al., 1988; Nguyen et al., 1994).

Given that polyribosomes were located near the base of many spines (Steward and Levy, 1982), it has been proposed that local protein synthesis could be a mechanism of providing individual synapses with newly synthesized molecules. By now numerous studies have demonstrated that mRNAs of various proteins could be targeted into dendrites including Arc1 (Link et al., 1995), Calmodulin (Berry and Brown, 1996), CamKII (Burgin et al., 1990), NMDAR (Gazzaley et al., 1997), MAP2 (Garner et al., 1988), and BDNF (Righi et al., 2000).

Locally synthesized proteins have been shown to contribute to NMDAR-LTP in the hippocampus. Isolation of synapses between neurons in area CA3 and CA1 from their pre- and postsynaptic cell bodies did not impair BDNF-induced synaptic potentiation which, however, could be blocked by protein synthesis inhibitors (Kang and Schuman, 1996). Electron microscopy studies documented that polyribosomes translocated from spine shafts into spine heads after LTP and that PSD-size was increased in those spines (Ostroff et al., 2002). This confirmed that LTP activates protein synthesis and that new structural proteins can increase synapse (and probably spine) size.

The functional significance of activity-dependent protein synthesis is still unclear. Although it is reasonable to assume that changes in spine morphology require additional proteins, LTP-induced spine formation has been observed to occur already during early-phase LTP, which is independent of protein synthesis. Apparently, formation of new spines does not directly rely on additionally synthesized proteins. Maybe reserve pools of proteins exist at synapses, which can rapidly supply new proteins and additional synthesis is required only later to replenish the pools.

Similarly, if LTD induces morphological changes one would expect that protein synthesis either would be needed to supply the new structures with proteins or promote the degradation of existing proteins. However, the finding that the retraction of small spines during NMDAR-LTD was independent of protein synthesis suggests that degradation machinery pre-exists at synapses, which suffices to remove small structures like stubby

spines. Only the degradation of large spines might require synthesis of additional proteins.

5. Summary

The aim of the present study was to investigate morphological plasticity in hippocampal organotypic slice cultures (Gähwiler cultures) and analyze the effect of NMDAR- and mGluR-LTD on spine morphology.

Using two-photon-laser microscopy, fluorescently labeled neurons were imaged at different time points following culture preparation. Image analysis revealed that Gähwiler cultures underwent extensive structural reorganization following the culture preparation and that morphological stability is achieved after approximately two weeks in culture. Cultures at this age and beyond exhibited a high degree of morphological stability and were used to study the effect of LTD on spine morphology.

NMDAR-LTD was induced and recorded by local stimulation of Schaffer collaterals and intracellular recordings of EPSPs. Individual neurons were filled with the fluorescent dye calcein via the recording electrode and imaged before and up until 60 min following the induction of NMDAR-LTD.

To quantify spine changes, spines were grouped into four categories according to their size. Morphological changes for one or two categories were classified as 1- and 2-category changes, respectively.

Image analysis by an unbiased observer did not yield evidence for NMDAR-LTD to lead to increased numbers of spines switching two or more categories within 60 min after LTD induction. Instead, a significantly higher number of small, stubby spines was lost during NMDAR-LTD than under control condition (category 1? 0). No such effect was observed for other spine categories.

To study the effect of mGluR-dependent LTD (mGluR-LTD) on spine morphology, mGluR-LTD was induced by bath-application of the mGluR-agonist DHPG. Intracellular recordings from individual neurons were performed and the morphology of the neurons was imaged before and up until 60 min following the induction of mGluR-LTD.

The overall number of 1- and 2-category changes was very small in comparison with the overall number of spines and no clear difference in the number of morphological changes between the mGluR and the control experiments could be detected. Therefore, there was

no evidence for mGluR-LTD to influence spine morphology within the analyzed time period after LTD induction.

To study the role of protein synthesis during NMDAR- and mGluR-LTD, extracellular recordings from area CA1 neurons were performed. Either form of LTD was induced and recorded for 60 min in the presence of the protein synthesis inhibitors anisomycin and cycloheximide. The levels of synaptic depression were found to be unaffected by the application of the inhibitors, suggesting that neither NMDAR- nor mGluR-LTD depended on protein synthesis in Gähwiler cultures within 60 min after LTD induction.

Taken together, morphological changes of dendritic spines appeared to be rare events in Gähwiler cultures. Neither NMDAR- nor mGluR-LTD induced major morphological changes of dendritic spines in the hippocampus. Only NMDAR-LTD was found to cause retraction of small spines. These results suggest that in this culture system dendritic spines are stable structures and that LTD induces morphological changes especially in small spines.

To circumvent the problem of culturing artifacts, future studies will have to confirm these results also in the intact brain. As long-term synaptic plasticity is generally believed to be the cellular basis of learning and memory, this would allow conclusions about the role of morphological changes in learning and memory processes in the brain.

6. References

Abraham WC, Mason-Parker SE, Bear MF, Webb S, Tate WP (2001) Heterosynaptic metaplasticity in the hippocampus in vivo: a BCM-like modifiable threshold for LTP. Proc Natl Acad Sci USA 98: 10924-10929.

Aramori I, Nakanishi S (1992) Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. Neuron 8: 757-765.

Artola A, Brocher S, Singer W (1990) Different voltage-dependent thresholds for inducing long-term depression and long-term potentiation in slices of rat visual cortex. Nature 347: 69-72.

Banke TG, Bowie D, Lee H, Huganir RL, Schousboe A, Traynelis SF (2000) Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. J Neurosci 20: 89-102.

Barde YA (1989) Trophic factors and neuronal survival. Neuron 2: 1525-1534.

Barria A, Müller D, Derkach V, Griffith LC, Soderling TR (1997a) Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. Science 276: 2042-2045.

Barria A, Müller D, Derkach V, Griffith LC, Soderling TR (1997b) Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. Science 276: 2042-2045.

Barrionuevo G, Schottler F, Lynch G (1980) The effects of repetitive low frequency stimulation on control and "potentiated" synaptic responses in the hippocampus. Life Sci 27: 2385-2391.

Bashir ZI, Bortolotto ZA, Davies CH, Berretta N, Irving AJ, Seal AJ, Henley JM, Jane DE, Watkins JC, Collingridge GL (1993) Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. Nature 363: 347-350.

Baude A, Nusser Z, Roberts JD, Mulvihill E, McIlhinney RA, Somogyi P (1993) The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 11: 771-787.

Bear MF (1995) Mechanism for a sliding synaptic modification threshold. Neuron 15: 1-4.

Becker JT, Walker JA, Olton DS (1980) Neuroanatomical bases of spatial memory. Brain Res 200: 307-320.

Berretta N, Cherubini E (1998) A novel form of long-term depression in the CA1 area of the adult rat hippocampus independent of glutamate receptors activation. Eur J Neurosci 10: 2957-2963.

Berry FB, Brown IR (1996) CaM I mRNA is localized to apical dendrites during postnatal development of neurons in the rat brain. J Neurosci Res 43: 565-575.

Bi GQ, Poo MM (1998) Synaptic modifications in cultured hippocampal neurons: Dependence on spike timing, synaptic strength, and postsynaptic cell type. J Neurosci 18: 10464-10472.

Bienenstock EL, Cooper LN, Munro PW (1982) Theory for the development of neuron selectivity: orientation specificity and binocular interaction in visual cortex. J Neurosci 2: 32-48.

Bliss TVP, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol (Lond) 232: 331-356.

Bolshakov VY, Siegelbaum SA (1994) Postsynaptic induction and presynaptic expression of hippocampal long- term depression. Science 264: 1148-1152.

Bonhoeffer T (1996) Neurotrophins and activity-dependent development of the neocortex. Curr Opin Neurobiol 6: 119-126.

Bonhoeffer T, Yuste R (2002) Spine motility. Phenomenology, mechanisms, and function. Neuron 35: 1019.

Brandon JG, Coss RG (1982) Rapid dendritic spine stem shortening during one-trial learning: the honeybee's first orientation flight. Brain Res 252: 51-61.

Buchs PA, Müller D (1996) Induction of long-term potentiation is associated with major ultrastructural changes of activated synapses. Proc Natl Acad Sci USA 93: 8040-8045.

Burgin KE, Waxham MN, Rickling S, Westgate SA, Mobley WC, Kelly PT (1990) In situ hybridization histochemistry of Ca2+/calmodulin-dependent protein kinase in developing rat brain. J Neurosci 10: 1788-1798.

Cameron HA, Woolley CS, McEwen BS, Gould E (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56: 337-344.

Carroll RC, Lissin DV, von Zastrow M, Nicoll RA, Malenka RC (1999) Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. Nat Neurosci 2: 454-460.

Chang FL, Greenough WT (1984) Transient and enduring morphological correlates of synaptic activity and efficacy change in the rat hippocampal slice. Brain Res 309: 35-46.

Chicurel ME, Harris KM (1992) Three-dimensional analysis of the structure and composition of CA3 branched dendritic spines and their synaptic relationships with mossy fiber boutons in the rat hippocampus. J Comp Neurol 325: 169-182.

Collin C, Miyaguchi K, Segal M (1997) Dendritic spine density and LTP induction in cultured hippocampal slices. J Neurophysiol 77: 1614-1623.

Collingridge GL, Bliss TVP (1987) NMDA receptors - their role in long-term potentiation. Trends Neurosci 10(7): 288-293.

Comery TA, Shah R, Greenough WT (1995) Differential rearing alters spine density on medium-sized spiny neurons in the rat corpus striatum: evidence for association of morphological plasticity with early response gene expression. Neurobiol Learn Mem 63: 217-219.

Conn PJ, Pin JP (1997) Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37: 205-237.

Craig AM (1998) Culturing nerve cells. In: Transfecting cultured neurons (Banker G, Goslin K, eds), pp 79-111. Cambridge MA: MIT Press.

Dailey ME, Smith SJ (1996) The dynamics of dendritic structure in developing hippocampal slices. J Neurosci 16: 2983-2994.

Debanne D, Gähwiler BH, Thompson SM (1994) Asynchronous pre- and postsynaptic activity induces associative long- term depression in area CA1 of the rat hippocampus in vitro. Proc Natl Acad Sci USA 91: 1148-1152.

Debanne D, Gähwiler BH, Thompson SM (1998) Long-term synaptic plasticity between pairs of individual CA3 pyramidal cells in rat hippocampal slice cultures. J Physiol 507: 237-247.

Debanne D, Guerineau NC, Gähwiler BH, Thompson SM (1995) Physiology and pharmacology of unitary synaptic connections between pairs of cells in areas CA3 and CA1 of rat hippocampal slice cultures. J Neurophysiol 73: 1282-1294.

Denk W, Strickler JH, Webb WW (1990) Two-photon laser scanning fluorescence microscopy. Science 248: 73-76.

Derkach V, Barria A, Soderling TR (1999) Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3- hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proc Natl Acad Sci USA 96: 3269-3274.

Desai NS, Rutherford LC, Turrigiano GG (1999) Plasticity in the intrinsic excitability of cortical pyramidal neurons. Nat Neurosci 2: 515-520.

Desmond NL, Levy WB (1985) Granule cell dendritic spine density in the rat hippocampus varies with spine shape and location. Neurosci Lett 54: 219-224.

Desmond NL, Levy WB (1986) Changes in the postsynaptic density with long-term potentiation in the dentate gyrus. J Comp Neurol 253: 476-482.

Desmond NL, Levy WB (1990) Morphological correlates of long-term potentiation imply the modification of existing synapses, not synaptogenesis, in the hippocampal dentate gyrus. Synapse 5: 139-143.

Dingledine R (1983) N-methyl aspartate activates voltage-dependent calcium conductance in rat hippocampal pyramidal cells. J Physiol 343: 385-405.

Dudek SM, Bear MF (1992) Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. Proc Natl Acad Sci USA 89: 4363-4367.

Dudek SM, Bear MF (1993) Bidirectional long-term modification of synaptic effectiveness in the adult and immature hippocampus. J Neurosci 13: 2910-2918.

Dunaevsky A, Blazeski R, Yuste R, Mason C (2001) Spine motility with synaptic contact. Nat Neurosci 4: 685-686.

Dunaevsky A, Tashiro A, Majewska A, Mason C, Yuste R (1999) Developmental regulation of spine motility in the mammalian central nervous system. Proc Natl Acad Sci USA 96: 13438-13443.

Eichenbaum H, Fagan A, Cohen NJ (1986) Normal olfactory discrimination learning set and facilitation of reversal learning after medial-temporal damage in rats: implications for an account of preserved learning abilities in amnesia. J Neurosci 6: 1876-1884.

Engert F, Bonhoeffer T (1999) Dendritic spine changes associated with hippocampal long-term synaptic plasticity. Nature 399: 66-70.

Federmeier K, Kleim IA, Anderson BJ, Greenough WT (1994) Formation of double synapses in the cerebellar cortex of the rat following motor learning. Soc Neurosci Abstr 20: 1435.

Fiala JC, Allwardt B, Harris KM (2002) Dendritic spines do not split during hippocampal LTP or maturation. Nat Neurosci 5: 297-298.

Fiala JC, Feinberg M, Popov V, Harris KM (1998) Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. J Neurosci 18: 8900-8911.

Fifkova E, Van Harreveld A (1977) Long-lasting morphological changes in dendritic spines of dentate granular cells following stimulation of the entorhinal area. J Neurocytol 6: 211-230.

Fischer M, Kaech S, Knutti D, Matus A (1998) Rapid actin-based plasticity in dendritic spines. Neuron 20: 847-854.

Fischer M, Kaech S, Wagner U, Brinkhaus H, Matus A (2000) Glutamate receptors regulate actin-based plasticity in dendritic spines. Nat Neurosci 3: 887-894.

Fitzjohn SM, Palmer MJ, May JE, Neeson A, Morris SA, Collingridge GL (2001) A characterisation of long-term depression induced by metabotropic glutamate receptor activation in the rat hippocampus in vitro. J Physiol 537: 421-430.

Frey U, Krug M, Reymann KG, Matthies H (1988) Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro. Brain Res 452: 57-65.

Frolov I, Hoffman TA, Pragai BM, Dryga SA, Huang HV, Schlesinger S, Rice CM (1996) Alphavirus-based expression vectors: strategies and applications. Proc Natl Acad Sci USA 93: 11371-11377.

Frotscher M, Gähwiler BH (1988) Synaptic organization of intracellularly stained CA3 pyramidal neurons in slice cultures of rat hippocampus. Neuroscience 24: 541-551.

Gähwiler BH (1981) Organotypic monolayer cultures of nervous tissue. J Neurosci Methods 4: 329-342.

Gähwiler BH, Thompson S, McKinney RA (1998) Organotypic slice cultures of neural tissue. In: Culturing nerve cells (Banker G, Goslin K, eds), pp 462-498. Cambridge, MA, USA: MIT Press.

Garner CC, Tucker RP, Matus A (1988) Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. Nature 336: 674-677.

Gärtner A, Shostak Y, Hackel N, Ethell IM, Thoenen H (2000) Ultrastructural identification of storage compartments and localization of activity-dependent secretion of neurotrophin 6 in hippocampal neurons. Mol Cell Neurosci 15: 215-234.

Gazzaley AH, Benson DL, Huntley GW, Morrison JH (1997) Differential subcellular regulation of NMDAR1 protein and mRNA in dendrites of dentate gyrus granule cells after perforant path transection. J Neurosci 17: 2006-2017.

Geinisman Y, Berry RW, Disterhoft JF, Power JM, Van der Zee EA (2001) Associative learning elicits the formation of multiple-synapse boutons. J Neurosci 21: 5568-5573.

Globus A, Scheibel AB (1967) The effect of visual deprivation on cortical neurons: a Golgi study. Exp Neurol 19: 331-345.

Grutzendler J, Kasthuri N, Gan WB (2002) Long-term dendritic spine stability in the adult cortex. Nature 420: 812-816.

Halpain S, Hipolito A, Saffer L (1998) Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. J Neurosci 18: 9835-9844.

Hansel C, Linden DJ (2000) Long-term depression of the cerebellar climbing fiber-Purkinje neuron synapse. Neuron 26: 473-482.

Harris KM (1999) Structure, development, and plasticity of dendritic spines. Curr Opin Neurobiol 9: 343-348.

Harris KM, Jensen FE, Tsao B (1992) Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. J Neurosci 12: 2685-2705.

Harris KM, Kater SB (1994) Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. Annu Rev Neurosci 17: 341-371.

Harris KM, Stevens JK (1989) Dendritic spines of CA 1 pyramidal cells in the rat hippocampus: serial electron microscopy with reference to their biophysical characteristics. J Neurosci 9: 2982-2997.

Hebb DO (1949) The organization of behavior. A neuropsychological theory. New York: Wiley.

Heynen AJ, Abraham WC, Bear MF (1996) Bidirectional modification of CA1 synapses in the adult hippocampus in vivo. Nature 381: 163-166.

Hirsch JC, Crepel F (1991) Blockade of NMDA receptors unmasks a long-term depression in synaptic efficacy in rat prefrontal neurons in vitro. Exp Brain Res 85: 621-624.

Holthoff K, Tsay D, Yuste R (2002) Calcium dynamics of spines depend on their dendritic location. Neuron 33: 425-437.

Horch HW, Kruttgen A, Portbury SD, Katz LC (1999) Destabilization of cortical dendrites and spines by BDNF. Neuron 23: 353-364.

Hosokawa T, Rusakov DA, Bliss TV, Fine A (1995) Repeated confocal imaging of individual dendritic spines in the living hippocampal slice: evidence for changes in length and orientation associated with chemically induced LTP. J Neurosci 15: 5560-5573.

Huber KM, Kayser MS, Bear MF (2000) Role for rapid dendritic protein synthesis in hippocampal mGluR- dependent long-term depression. Science 288: 1254-1257.

Husi H, Ward MA, Choudhary JS, Blackstock WP, Grant SG (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. Nat Neurosci 3: 661-669.

Isaac JT, Nicoll RA, Malenka RC (1995) Evidence for silent synapses: implications for the expression of LTP. Neuron 15: 427-434.

Jones EG, Powell TP (1969) Morphological variations in the dendritic spines of the neocortex. J Cell Sci 5: 509-529.

Jones TA, Klintsova AY, Kilman VL, Sirevaag AM, Greenough WT (1997) Induction of multiple synapses by experience in the visual cortex of adult rats. Neurobiol Learn Mem 68: 13-20.

Kameyama K, Lee HK, Bear MF, Huganir RL (1998) Involvement of a postsynaptic protein kinase A substrate in the expression of homosynaptic long-term depression. Neuron 21: 1163-1175.

Kandler K, Katz LC, Kauer JA (1998) Focal photolysis of caged glutamate produces long-term depression of hippocampal glutamate receptors. Nat Neurosci 1: 119-123.

Kang H, Schuman EM (1995) Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 267: 1658-1662.

Kang H, Schuman EM (1996) A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. Science 273: 1402-1406.

Kauderer BS, Kandel ER (2000) Capture of a protein synthesis-dependent component of long-term depression. Proc Natl Acad Sci USA 97: 13342-13347.

Kemp N, Bashir ZI (1997) NMDA receptor-dependent and -independent long-term depression in the CA1 region of the adult rat hippocampus in vitro. Neuropharmacology 36: 397-399.

Kemp N, Bashir ZI (1999) Induction of LTD in the adult hippocampus by the synaptic activation of AMPA/kainate and metabotropic glutamate receptors. Neuropharmacology 38: 495-504.

Kemp N, McQueen J, Faulkes S, Bashir ZI (2000) Different forms of LTD in the CA1 region of the hippocampus: role of age and stimulus protocol. Eur J Neurosci 12: 360-366.

Kinoshita S, Yasuda H, Taniguchi N, Katoh-Semba R, Hatanaka H, Tsumoto T (1999) Brain-derived neurotrophic factor prevents low-frequency inputs from inducing long-term depression in the developing visual cortex. J Neurosci 19: 2122-2130.

Kirov SA, Harris KM (1999) Dendrites are more spiny on mature hippocampal neurons when synapses are inactivated. Nat Neurosci 2: 878-883.

Kirov SA, Sorra KE, Harris KM (1999) Slices have more synapses than perfusion-fixed hippocampus from both young and mature rats. J Neurosci 19: 2876-2886.

Korkotian E, Segal M (2000) Structure-function relations in dendritic spines: is size important? Hippocampus 10: 587-595.

Korkotian E, Segal M (2001) Regulation of dendritic spine motility in cultured hippocampal neurons. J Neurosci 21: 6115-6124.

Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T (1995) Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc Natl Acad Sci USA 92: 8856-8860.

Korte M, Griesbeck O, Gravel C, Carroll P, Staiger V, Thoenen H, Bonhoeffer T (1996) Virus-mediated gene transfer into hippocampal CA1 region restores long-term potentiation in brain-derived neurotrophic factor mutant mice. Proc Natl Acad Sci USA 93: 12547-12552.

Lee HK, Barbarosie M, Kameyama K, Bear MF, Huganir RL (2000) Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. Nature 405: 955-959.

Lee HK, Kameyama K, Huganir RL, Bear MF (1998) NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus. Neuron 21: 1151-1162.

Lee KS, Schottler F, Oliver M, Lynch G (1980) Brief bursts of high-frequency stimulation produce two types of structural change in rat hippocampus. J Neurophysiol 44: 247-258.

Lendvai B, Stern EA, Chen B, Svoboda K (2000) Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. Nature 404: 876-881.

Leuner B, Falduto J, Shors TJ (2003) Associative memory formation increases the observation of dendritic spines in the hippocampus. J Neurosci 23: 659-665.

Liao D, Hessler NA, Malinow R (1995) Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. Nature 375: 400-404.

Link W, Konietzko U, Kauselmann G, Krug M, Schwanke B, Frey U, Kuhl D (1995) Somatodendritic expression of an immediate early gene is regulated by synaptic activity. Proc Natl Acad Sci USA 92: 5734-5738.

Lisman J (1989) A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. Proc Natl Acad Sci USA 86: 9574-9578.

Lujan R, Nusser Z, Roberts JD, Shigemoto R, Somogyi P (1996) Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Eur J Neurosci 8: 1488-1500.

Lujan R, Roberts JD, Shigemoto R, Ohishi H, Somogyi P (1997) Differential plasma membrane distribution of metabotropic glutamate receptors mGluR1 alpha, mGluR2 and mGluR5, relative to neurotransmitter release sites. J Chem Neuroanat 13: 219-241.

Luthi A, Chittajallu R, Duprat F, Palmer MJ, Benke TA, Kidd FL, Henley JM, Isaac JT, Collingridge GL (1999) Hippocampal LTD expression involves a pool of AMPARs regulated by the NSF-GluR2 interaction. Neuron 24: 389-399.

Lynch GS, Dunwiddie T, Gribkoff V (1977) Heterosynaptic depression: a postsynaptic correlate of long-term potentiation. Nature 266: 737-739.

Majewska A, Brown E, Ross J, Yuste R (2000) Mechanisms of calcium decay kinetics in hippocampal spines: role of spine calcium pumps and calcium diffusion through the spine neck in biochemical compartmentalization. J Neurosci 20: 1722-1734.

Malenka RC, Kauer JA, Zucker RS, Nicoll RA (1988) Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. Science 242: 81-84.

Maletic-Savatic M, Malinow R, Svoboda K (1999) Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. Science 283: 1923-1927.

Mammen AL, Kameyama K, Roche KW, Huganir RL (1997) Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole4- propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. J Biol Chem 272: 32528-32533.

Mannaioni G, Marino MJ, Valenti O, Traynelis SF, Conn PJ (2001) Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function. J Neurosci 21: 5925-5934.

Marrs GS, Green SH, Dailey ME (2001) Rapid formation and remodeling of postsynaptic densities in developing dendrites. Nat Neurosci 4: 1006-1013.

Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M, Kasai H (2001) Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. Nat Neurosci 4: 1086-1092.

Matus A (2000) Actin-based plasticity in dendritic spines. Science 290: 754-758.

Mayer ML, Westbrook GL, Guthrie PB (1984) Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurons. Nature 309: 261-263.

Mayford M, Wang J, Kandel ER, O'Dell TJ (1995) CaMKII regulates the frequency-response function of hippocampal synapses for the production of both LTD and LTP. Cell 81: 891-904.

McKinney RA, Capogna M, Durr R, Gähwiler BH, Thompson SM (1999) Miniature synaptic events maintain dendritic spines via AMPA receptor activation . Nat Neurosci 2: 44-49.

Migaud M, Charlesworth P, Dempster M, Webster LC, Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RG, Morrison JH, O'Dell TJ, Grant SG (1998) Enhanced long-

term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature 396: 433-439.

Morris RG, Anderson E, Lynch GS, Baudry M (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature 319: 774-776.

Morris RG, Garrud P, Rawlins JN, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297: 681-683.

Moser EI, Krobert KA, Moser MB, Morris RG (1998) Impaired spatial learning after saturation of long-term potentiation. Science 281: 2038-2042.

Moser MB, Trommald M, Egeland T, Andersen P (1997) Spatial training in a complex environment and isolation alter the spine distribution differently in rat CA1 pyramidal cells. J Comp Neurol 380: 373-381.

Mulkey RM, Endo S, Shenolikar S, Malenka RC (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature 369: 486-488.

Mulkey RM, Herron CE, Malenka RC (1993) An essential role for protein phosphatases in hippocampal long-term depression. Science 261: 1051-1055.

Mulkey RM, Malenka RC (1992) Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of the hippocampus. Neuron 9: 967-975.

Nakanishi S, Masu M, Bessho Y, Nakajima Y, Hayashi Y, Shigemoto R (1994) Molecular diversity of glutamate receptors and their physiological functions. EXS 71: 71-80.

Nguyen PV, Abel T, Kandel ER (1994) Requirement of a critical period of transcription for induction of a late phase of LTP. Science 265: 1104-1107.

Nusser Z, Lujan R, Laube G, Roberts JD, Molnar E, Somogyi P (1998) Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. Neuron 21: 545-559.

O'Brien RJ, Kamboj S, Ehlers MD, Rosen KR, Fischbach GD, Huganir RL (1998) Activity-dependent modulation of synaptic AMPA receptor accumulation. Neuron 21: 1067-1078.

O'Connor JJ, Rowan MJ, Anwyl R (1994) Long-lasting enhancement of NMDA receptor-mediated synaptic transmission by metabotropic glutamate receptor activation. Nature 367: 557-559.

Okabe S, Miwa A, Okado H (2001) Spine formation and correlated assembly of presynaptic and postsynaptic molecules. J Neurosci 21: 6105-6114.

Oliet SH, Malenka RC, Nicoll RA (1997) Two distinct forms of long-term depression coexist in CA1 hippocampal pyramidal cells. Neuron 18: 969-982.

Ostroff LE, Fiala JC, Allwardt B, Harris KM (2002) Polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP in developing rat hippocampal slices. Neuron 35: 535-545.

Palmer MJ, Irving AJ, Seabrook GR, Jane DE, Collingridge GL (1997) The group I mGlu receptor agonist DHPG induces a novel form of LTD in the CA1 region of the hippocampus. Neuropharmacology 36: 1517-1532.

Parnass Z, Tashiro A, Yuste R (2000) Analysis of spine morphological plasticity in developing hippocampal pyramidal neurons. Hippocampus 10: 561-568.

Parnavelas JG, Globus A, Kaups P (1973) Continuous illumination from birth affects spine density of neurons in the visual cortex of the rat. Exp Neurol 40: 742-747.

Parnavelas JG, Lynch G, Brecha N, Cotman CW, Globus A (1974) Spine loss and regrowth in hippocampus following deafferentation. Nature 248: 71-73.

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16: 1137-1145.

Pokorny J, Yamamoto T (1981) Postnatal ontogenesis of hippocampal CA1 area in rats. II. Development of ultrastructure in stratum lacunosum and moleculare. Brain Res Bull 7: 121-130.

Polnau, DG (2003) Molecular and morphological mechanisms underlying acitivity dependent synaptic plasticity in the hippocampus. Thesis/ Dissertation MPI of Neurobiology.

Popov VI, Bocharova LS (1992) Hibernation-induced structural changes in synaptic contacts between mossy fibres and hippocampal pyramidal neurons. Neuroscience 48: 53-62.

Ramón y Cajal S (1891) Significación fisiológica de las expansiones protoplásmicas y nerviosas de la sustancia gris. Rev Cie Med Barcelona 22: 23.

Ramón y Cajal S (1893) Neue Darstellung vom Histologischen Bau des Centralnervensystems. Arch Anat Entwickl 319-428.

Rampon C, Tang YP, Goodhouse J, Shimizu E, Kyin M, Tsien JZ (2000) Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice. Nat Neurosci 3: 238-244.

Righi M, Tongiorgi E, Cattaneo A (2000) Brain-Derived Neurotrophic Factor (BDNF) Induces Dendritic Targeting of BDNF and Tyrosine Kinase B mRNAs in Hippocampal

Neurons through a Phosphatidylinositol-3 Kinase-Dependent Pathway. J Neurosci 20: 3165-3174.

Robain O, Barbin G, Billette d, V, Jardin L, Jahchan T, Ben Ari Y (1994) Development of mossy fiber synapses in hippocampal slice culture. Brain Res Dev Brain Res 80: 244-250.

Roberson ED, English JD, Adams JP, Selcher JC, Kondratick C, Sweatt JD (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. J Neurosci 19: 4337-4348.

Routtenberg A, Colley P, Linden D, Lovinger D, Murakami K, Sheu FS (1986) Phorbol ester promotes growth of synaptic plasticity. Brain Res 378: 374-378.

Rusakov DA, Richter-Levin G, Stewart MG, Bliss TV (1997) Reduction in spine density associated with long-term potentiation in the dentate gyrus suggests a spine fusion-and-branching model of potentiation. Hippocampus 7: 489-500.

Sabatini BL, Maravall M, Svoboda K (2001) Ca(2+) signaling in dendritic spines. Curr Opin Neurobiol 11: 349-356.

Schikorski T, Stevens CF (1997) Quantitative ultrastructural analysis of hippocampal excitatory synapses. J Neurosci 17: 5858-5867.

Schoepp DD, Goldsworthy J, Johnson BG, Salhoff CR, Baker SR (1994) 3,5-dihydroxyphenylglycine is a highly selective agonist for phosphoinositide-linked metabotropic glutamate receptors in the rat hippocampus. J Neurochem 63: 769-772.

Segal M (1995) Morphological alterations in dendritic spines of rat hippocampal neurons exposed to N-methyl-D-aspartate. Neurosci Lett 193: 73-76.

Segal M, Korkotian E, Murphy DD (2000) Dendritic spine formation and pruning: common cellular mechanisms? Trends Neurosci 23: 53-57.

Shepherd GM (1996) The dendritic spine: a multifunctional integrative unit. J Neurophysiol 75: 2197-2210.

Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, Malinow R (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. Science 284: 1811-1816.

Shors TJ, Chua C, Falduto J (2001) Sex differences and opposite effects of stress on dendritic spine density in the male versus female hippocampus. J Neurosci 21: 6292-6297.

Sik A, Tamamaki N, Freund TF (1993) Complete axon arborization of a single CA3 pyramidal cell in the rat hippocampus, and its relationship with postsynaptic parvalbumin- containing interneurons. Eur J Neurosci 5: 1719-1728.

Skeberdis VA, Lan J, Opitz T, Zheng X, Bennett MV, Zukin RS (2001) mGluR1-mediated potentiation of NMDA receptors involves a rise in intracellular calcium and activation of protein kinase C. Neuropharmacology 40: 856-865.

Snyder EM, Philpot BD, Huber KM, Dong X, Fallon JR, Bear MF (2001) Internalization of ionotropic glutamate receptors in response to mGluR activation. Nat Neurosci 4: 1079-1085.

Sorra KE, Fiala JC, Harris KM (1998) Critical assessment of the involvement of perforations, spinules, and spine branching in hippocampal synapse formation. J Comp Neurol 398: 225-240.

Sorra KE, Harris KM (1998) Stability in synapse number and size at 2 hr after long-term potentiation in hippocampal area CA1. J Neurosci 18: 658-671.

Sorra KE, Harris KM (2000) Overview on the structure, composition, function, development, and plasticity of hippocampal dendritic spines. Hippocampus 10: 501-511.

Stanton PK, Chattarji S, Sejnowski TJ (1991) 2-Amino-3-phosphonopropionic acid, an inhibitor of glutamate-stimulated phosphoinositide turnover, blocks induction of homosynaptic long-term depression, but not potentiation, in rat hippocampus. Neurosci Lett 127: 61-66.

Steward O, Levy WB (1982) Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. J Neurosci 2: 284-291.

Stoppini L, Buchs PA, Müller D (1991) A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37: 173-182.

Svoboda K, Tank DW, Denk W (1996) Direct measurement of coupling between dendritic spines and shafts. Science 272: 716-719.

Swindale N (1981) Dendritic spines only connect. Trends Neurosci 4: 240-241.

Thiels E, Norman ED, Barrionuevo G, Klann E (1998) Transient and persistent increases in protein phosphatase activity during long-term depression in the adult hippocampus in vivo. Neuroscience 86: 1023-1029.

Toni N, Buchs PA, Nikonenko I, Bron CR, Müller D (1999) LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. Nature 402: 421-425.

Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR, Welker E, Svoboda K (2002) Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature 420: 788-794.

Trommald M, Hulleberg G, Andersen P (1996) Long-term potentiation is associated with new excitatory spine synapses on rat dentate granule cells. Learn Mem 3: 218-228.

Turrigiano GG (1999) Homeostatic plasticity in neuronal networks: the more things change, the more they stay the same. Trends Neurosci 22: 221-227.

Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB (1998) Activity-dependent scaling of quantal amplitude in neocortical neurons. Nature 391: 892-896.

Van Harreveld A, Fifkova E (1975) Swelling of dendritic spines in the fascia dentata after stimulation of the perforant fibers as a mechanism of post-tetanic potentiation. Exp Neurol 49: 736-749.

Vees AM, Micheva KD, Beaulieu C, Descarries L (1998) Increased number and size of dendritic spines in ipsilateral barrel field cortex following unilateral whisker trimming in postnatal rat. J Comp Neurol 400: 110-124.

Vicario-Abejon C, Collin C, McKay RD, Segal M (1998) Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. J Neurosci 18: 7256-7271.

Volfovsky N, Parnas H, Segal M, Korkotian E (1999) Geometry of dendritic spines affects calcium dynamics in hippocampal neurons: theory and experiments. J Neurophysiol 82: 450-462.

Wang SJ, Gean PW (1999) Long-term depression of excitatory synaptic transmission in the rat amygdala. J Neurosci 19: 10656-10663.

Weiler IJ, Greenough WT (1993) Metabotropic glutamate receptors trigger postsynaptic protein synthesis. Proc Natl Acad Sci USA 90: 7168-7171.

Wickens J (1988) Electrically coupled but chemically isolated synapses: dendritic spines and calcium in a rule for synaptic modification. Prog Neurobiol 31: 507-528.

Xiao MY, Zhou Q, Nicoll RA (2001) Metabotropic glutamate receptor activation causes a rapid redistribution of AMPA receptors. Neuropharmacology 41: 664-671.

Yuste R, Bonhoeffer T (2001) Morphological changes in dendritic spines associated with long-term synaptic plasticity. Annu Rev Neurosci 24: 1071-1089.

Yuste R, Denk W (1995) Dendritic spines as basic functional units of neuronal integration. Nature 375: 682-684.

Zakharenko S, Zablow L, Siegelbaum S (2002) Altered Presynaptic Vesicle Release and Cycling during mGluR-Dependent LTD. Neuron 35: 1099.

Zimmer J, Gähwiler BH (1984) Cellular and connective organization of slice cultures of the rat hippocampus and fascia dentata. J Comp Neurol 228: 432-446.

Ziv NE, Smith SJ (1996) Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. Neuron 17: 91-102.

Abbreviations

AMPAR:	a–amino-3-hydroxy-5-methylisoxazole-4-propionate receptors; subtype of glutamate receptors
cat:	Category
DIV	Days *in vitro*
eGFP:	Enhanced green fluorescent protein
EPSPs:	Excitatory postsynaptic potentials
fEPSPs:	Field excitatory postsynaptic potentials
DHPG:	(RS)-3,5- Dihydroxyphenylglycine; agonist of group 1 metabotropic glutamate receptors
LFS:	Low-frequency stimulation; induction protocol for NMDAR-dependent long-term depression
LTD:	Long-term depression
LTP:	Long-term potentiation
mGluR-LTD:	metabotropic glutamate receptor dependent long-term depression
NMDAR:	N-methyl-D-aspartate-receptors; subtype of glutamate receptors
NMDAR-LTD:	NMDAR-dependent long-term depression
PMT:	Photomultiplier
SEM	Standard error of the mean
TPLM:	Two-photon laser microscopy

Curriculum vitae

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1991-1992	Studies of physics at the Technische Universität (München)
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