Synaptic Plasticity in Mature Cultured Hippocampal-Entorhinal Cortex Slices: Activity-Dependent Regulation of cAMP Response-Element Binding Protein

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Jill K. Leutgeb

ABSTRACT

Cultured hippocampal neurons and organotypic slices have been useful for investigating long-lasting plasticity beyond the time limit of acutely prepared slices. However, difficulties with culturing adult neurons have restricted such studies to preparations from embryonic, perinatal, and juvenile tissue. Immature hippocampal cultures not only differ in the anatomical organization and maturity of their neurons from adult tissue, but also in the mechanisms for the induction and expression of long-term potentiation (LTP). This study provides evidence that mature hippocampal cultures can retain electrophysiological properties required for long-term plasticity for several weeks in vitro. Introducing improved methods for culturing and maintaining hippocampal-entorhinal cortex slices from young adult rats (P25-30) resulted in cultures for use in long-term electrophysiological investigations. The electrophysiological properties and, in particular, the induction of LTP in mature organotypic slices were highly sensitive to dissection and tissue culture techniques. Using the modified preparation and culture protocols, cultured mature slices maintained an intact and functional trisynaptic cascade, synaptic function comparable to acute slices, as well as reliable long-term recording stability for at least 14 days in vitro. As in the adult hippocampus in vivo, LTP at the Schaffer-collateral-CA1 synapse could be induced by extracellular stimulation. Its induction was *N*-methyl-D-aspartate (NMDA) receptor dependent and its maintenance long-lasting (> 4 h). The development of mature slice cultures and protocols for LTP induction makes further studies investigating the mechanisms involved in the long-lasting maintenance of LTP feasible. For example, phosphorylation of the transcription factor cAMP-response element binding protein (CREB) has been implicated in synaptic plasticity and long-term memory, and its sustained activation has been proposed to be required for the maintenance of late-LTP (L-LTP). In the present work, the level of CREB

phosphorylation was determined for individual neurons in mature organotypic hippocampal slices after LTP was induced by stimulating the CA1 area. Confocal imaging was used to determine the ratio between nonphosphorylated and phosphorylated CREB (pCREB) revealing the extent of CREB phosphorylation at a single-cell resolution. The activation of CREB after LTP induction was compared to cAMP-activation after bath application of forskolin. An increase in cAMP by forskolin resulted in a persistent and uniform increase of the pCREB/CREB immunofluorescence ratio in the entire hippocampal principal neuron population. High-frequency tetanization (100Hz) in the CA1 area resulted in long-lasting LTP accompanied by a significant increase in the pCREB/CREB ratio, which continued to increase in parallel with the increased duration of LTP. Specific for CA1 cells following tetanization was a marked variability of CREB phosphorylation between adjacent cells throughout the duration of LTP. Only LTP-inducing stimuli translated synaptic input into varied degrees of CREB phosphorylation, and resulted in the continued increase of the proportion of nuclear CREB phosphorylation in parallel to the maintenance of long-lasting LTP irrespective of the initial level of activation. Activity-dependent CREB activation was specific for CA1 neurons, whereas CA3 and dentate neurons remained at baseline levels indicating that antidromic stimulation was not sufficient for inducing CREB phosphorylation. In addition, 100 Hz stimulation in the presence of an NMDA receptor antagonist resulted in a short-lasting posttetanic potentiation and an unchanged pCREB/CREB ratio revealing that both CREB phosphorylation and LTP induction in mature slices required NMDA receptor activation. This study supports the hypothesis that CREB may play a role in the late phases of LTP and provides evidence that molecular and electrophysiological plasticity can be studied in parallel in mature cultured tissue, which can be maintained in culture without a loss in hippocampal cell function or stability.

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1. Introduction

1.1. Synaptic Plasticity

The remarkable capacity of the brain to continually create and store endless volumes of memories is thought to rely on long-lasting, activity-dependent changes in the strength of synaptic connections between neurons. Ramon y Cajal (1894) first proposed that memory was stored as an anatomical change in the strength between neuronal connections. His classical descriptions of nervous system structure and function were the basis for the innovative theories put forth by the Canadian psychologist Donald Hebb in the late 1940s. He proposed the following idea to explain how synapses possibly change:

> "When an axon of cell A is near enough to excite cell B repeatedly or consistently takes place in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased" (Hebb, 1949, pg.62).

> "… any two cells or systems of cells that are repeatedly active at the same time will tend to become 'associated', so that activity in one facilitates activity in the other" (Hebb, 1949, pg. 70).

Support that mammalian synapses could undergo such a type of modification did not appear until 1973 when Timothy Bliss and Terje Lømo described a 'Hebbian' form of plasticity in the mammalian brain. In their groundbreaking study, Bliss and Lømo demonstrated in anesthetized rabbits that repeated high-frequency stimulation of the

perforant path in vivo, a fiber pathway to the dentate gyrus of the hippocampal formation, produced a long-lasting enhancement of the extracellularly recorded field potential, which they called long-term potentiation (LTP). The increased postsynaptic field potential, which was taken to reflect strengthened synapses between the perforant path and dentate granule cells, could last for hours in vitro and days and even weeks in the intact animal (Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973).

It is believed that understanding the mechanisms of LTP will uncover the processes involved in the formation and storage of memories, and lead to a greater understanding of physiological and molecular events resulting in learning and memory. LTP has been studied using a variety of methods, both in vitro and in vivo, in many different synapses throughout the brain of several species. The fact that LTP can be reliably generated in brain regions thought to constitute the core anatomical components of learning and memory (Hippocampal formation, Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973; Septum, Racine et al., 1983; Neocortex, Kirkwood et al., 1993; Heynen and Bear, 2001; and Amygdala, Yaniv et al., 2001) provides further evidence for its involvement in learning and memory. Considering the important role of the hippocampus in memory formation and taking advantage of its relatively simple circuitry and laminar organization has focused much of the experimental research on understanding LTP at the excitatory synapses of the hippocampal formation. The hippocampus appears to be involved in the acquisition and consolidation of long-term memories, including episodic memory and memories with spatial content (Milner et al., 1968; Smith and Milner, 1981; Kesner et al., 1989; Squire et al., 1993). Furthermore, many cellular and molecular processes that are necessary for hippocampal-dependent memory are also required for LTP induction and maintenance (Morris et al., 1986; Izquierdo et al., 1997; Morris et al., 2003).

1.2. Properties of Long-Term Potentiation in the Hippocampus

The hippocampal formation is an area of primitive cortex (i.e., allocortex) connected to the adjacent entorhinal cortex and other associative cortical areas (Fig. 1a). The hippocampal formation is composed of the subiculum, the hippocampus proper, and the dentate gyrus. Based on cytoarchitecture the hippocampus proper is further subdivided into four regions designated CA1-CA4 (cornu ammonis, or Ammon's horn), where CA1 is located proximal to the subiculum and CA4 close to the dentate gyrus. The basic anatomical connections of the hippocampal formation were first observed in the classical Golgi studies of Ramon y Cajal (1893) and Lorente de No (1933, 1934), but it was not until the early 1970's that physiological studies were combined with previous anatomical studies to provide a clearer description of the three-dimensional and laminar organization of the hippocampus (Andersen et al., 1971, 1977). Per Andersen et al. (1977) provided physiological evidence that the hippocampal formation is composed of three major excitatory pathways forming a trisynaptic cascade. The first major excitatory pathway is called the perforant pathway. This input to the hippocampal formation originates from layers two and three of the entorhinal cortex and passes through the subiculum to terminate in the molecular layer of the dentate gyrus. Granule cells in the dentate gyrus form the second excitatory pathway, the mossy fiber pathway, which projects to the proximal dendrites of pyramidal cells in the CA3 region of the hippocampus. In addition to sending recurrent collaterals to cells within CA3 and collaterals back to the granule cells, CA3 pyramidal neurons also send an excitatory projection, comprising the third excitatory pathway, to the pyramidal cells in the CA1 hippocampal region (i.e., Schaffer collateral fibers). The CA1 neurons, in turn, provide input to the subiculum as well as layers four and five of the entorhinal cortex (see Witter, 1993; Fig. 1b).

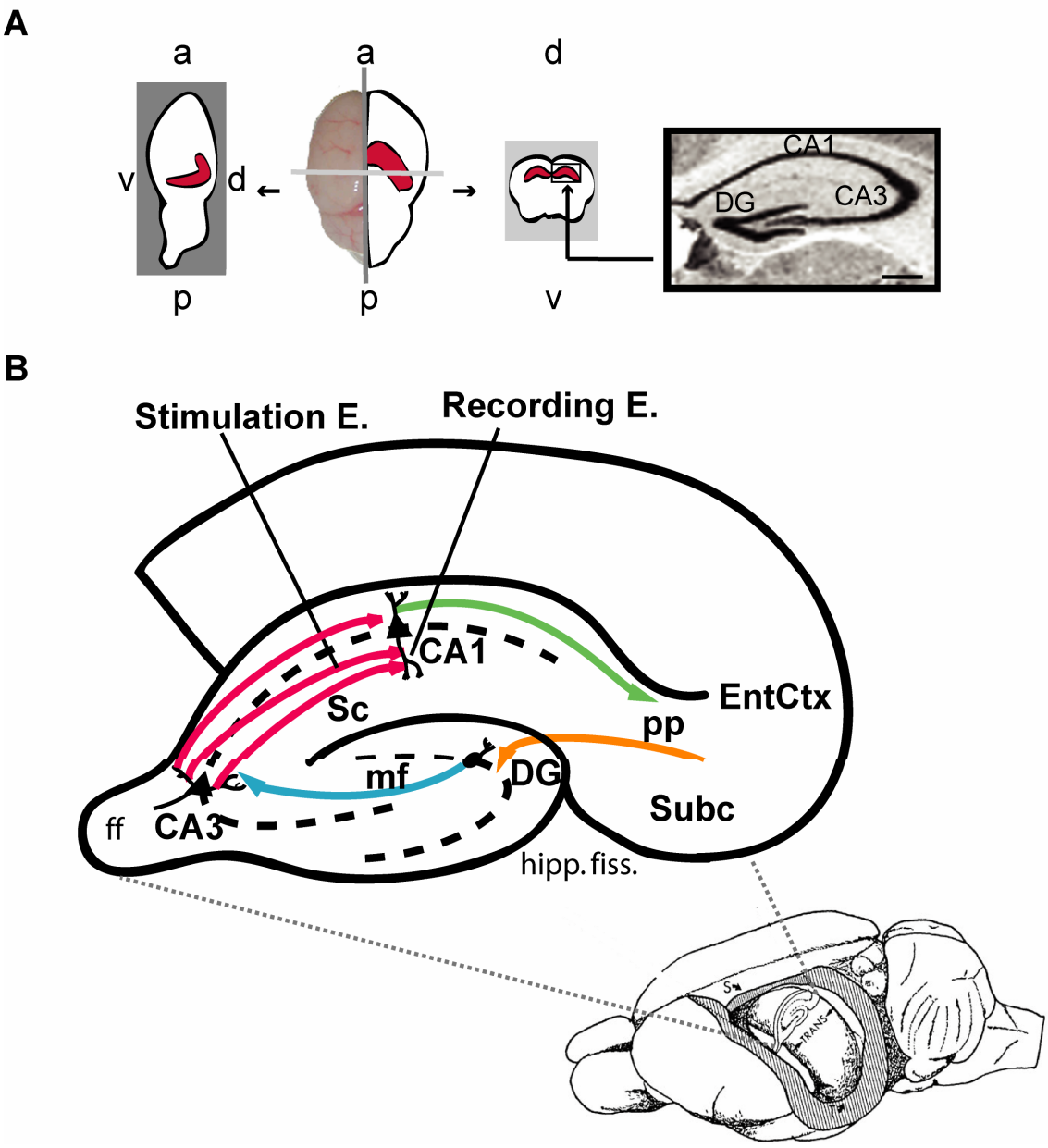

Figure 1. Reconstruction of the hippocampus (*red*) oriented within the rat brain. (A) Lines indicate the orientation of additional visual planes represented at the left and right respectively. The internal anatomy of the hippocampus is represented by a Nissl-stained coronal section (*right box*; Nissl-stained image adapted from Lu et al., 2001). (B) A schematic representation of the trisynaptic hippocampal cascade (illustrated in a hippocampal slice). The position of the hippocampal slice is shown in the rat brain drawn below (image adapted from Amaral and Witter, 1989). The electrode placement is shown for recording LTP evoked at the synapses of the Schaffer collateral input to the pyramidal neurons of the CA1 region. Scale bar: 600 μm. a, anterior; p, posterior; m, medial; l, lateral; d, dorsal; v, ventral; DG, dentate gyrus; ff, fimbria fornex; hipp. fiss., hippocampal fissure; mf, mossy fiber pathway; pp, perforant pathway; Subc, subiculum; sc, Schaffer collateral fibers; TRANS, transverse axis.

A commonly studied synapse in the hippocampus, and in the entire brain is the Schaffer collateral input to the pyramidal neurons of the CA1 region. LTP evoked at the synapses between the Schaffer collateral axons and the apical dendrites of CA1 pyramidal cells is characterized by three properties: cooperativity, associativity, and specificity. Cooperativity refers to the fact that the probability of inducing LTP increases with the number of stimulated afferents. Weak high-frequency stimulation was found to result in LTP less often than strong stimulation at the same frequency and duration presumably due to the recruitment of more axons at higher stimulation intensities, which 'cooperate' to trigger LTP (McNaughton et al., 1978). LTP in area CA1 is also associative as shown in preparations when two distinct axonal inputs converge on the same cell, where stimulation of a weak input (few stimulated afferents) only evokes LTP when coupled with stimulation of the strong input (many stimulated afferents). Or in other words, strong activation of one set of synapses can facilitate LTP at synapses on the same cell if both are activated during a specific time window (Barrionuevo and Brown, 1983; Levy and Steward, 1983). Finally, LTP in area CA1 is input-specific, meaning that LTP is only evoked at the inputs that receive high-frequency stimulation (Lynch et al., 1977; Kelso and Brown, 1986). Accordingly, LTP in area CA1 has been shown to closely follow the properties proposed by Donald Hebb. The first direct evidence that CA1 synapses were 'Hebbian' was provided by substituting the usual strong input with direct depolarization of the postsynaptic neuron (Kelso et al., 1986), and by showing that LTP induction in the CA1 region requires depolarization of the postsynaptic cell that coincided with activity in the presynaptic neuron (Gustafson and Wigström, 1988).

Different forms of LTP have also been shown for mammalian synapses, which in turn display differing properties including non-Hebbian mechanisms (reviewed in Bliss and Collingridge, 1993; Nicoll and Malenka, 1995; Morris and Frey, 1997; Bailey et al., 2000). For example, non-glutamatergic heterosynaptic inputs have been hypothesized to

enhance synaptic strengthening that was initially induced by homosynaptic mechanisms. It has been proposed that 'Hebbian' mechanisms are primarily responsible for short-term memories, whereas plasticity resulting from the heterosynaptic input is required for long-term memory (Frey et al., 1988; Frey and Morris, 1998). Synapse strengthening by a modulatory pathway would accordingly be necessary for prolonged synaptic plasticity and in particular protein synthesis dependent late-LTP (Frey et al. 1988; reviewed in Matthies et al., 1990; Frey and Morris, 1998; Bailey et al., 2000; Kendal et al., 2001).

1.3. Mechanisms for the Induction of Long-Term Potentiation

The *N*-methyl-D-aspartate (NMDA) receptor, which is a glutamate receptor subtype, has key molecular properties that convey 'Hebbian' synaptic plasticity. NMDA receptors are permeable to Ca^{2+}, the critical trigger for the induction of LTP, and its permeability depends on both pre- and postsynaptic events. Opening of the channel for the influx of Ca^{2+} and Na^{+} ions requires the binding of the neurotransmitter glutamate, which is released from the presynaptic cell. In addition, coincident and sufficient depolarization of the postsynaptic cell is needed to disassociate Mg^{2+} from its binding site within the NMDA receptor channel before allowing Ca^{2+} and Na^{+} entry into the cell. The depolarization is largely mediated by the activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors, which are co-localized with NMDA receptors on dendritic spines and are also activated by the binding of presynaptically released glutamate. However, it is the properties of the NMDA receptor that account for the induction of LTP through the influx of Ca^{2+} into the dendritic spine, which in turn activates Ca^{2+} dependent enzymes responsible for the induction of LTP (Fig. 2). The Hebbian properties of LTP can be fully explained by the behavior of the NMDA receptor voltage dependence, which allows for Ca^{2+} entry into the cell only when the presynaptic release

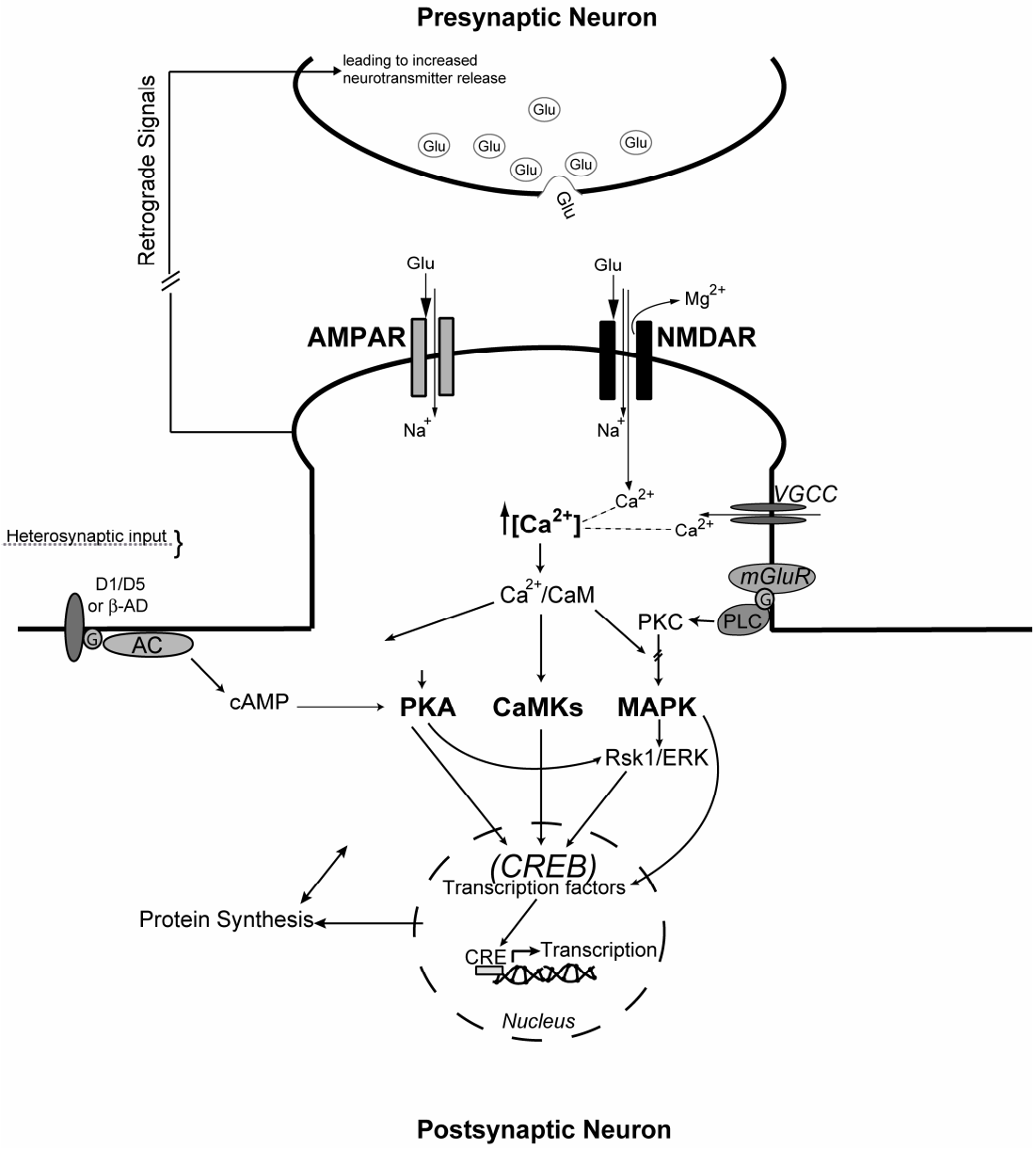

Figure 2. Events leading to LTP as well as CREB activation. The schematic depicts events in the pre- and postsynaptic spines following forced synaptic input by electrical stimulation. Tetanization of the presynaptic neuron causes glutamate (glu) to be released from the presynaptic bouton that acts on both AMPA and NMDA receptors. The NMDA receptor admits Ca^{2+} only after sufficient depolarization removes the Mg^{2+} block, the subsequent rise in $[Ca^{2+}]$ is the critical trigger for the induction of LTP. Various sources of Ca^{2+} in the postsynaptic spine are shown. The activation of second messenger cascades acting through their respective protein kinases affect multiple cellular processes such as the phosphorylation of CREB and result in the enhancement of synaptic transmission. Long-term alterations in synaptic strength (i.e., L-LTP) are achieved through regulation of protein synthesis. However, the direct role of CREB or other transcription factors has not been conclusively determined (see text for additional details). β-AD (β-adrenergic receptor); AC (adenyl cyclase); D1/D5 (D1/D5 dopamine receptor); G (G-protein); mGluR (metabotropic glutamate receptor); PLC (phospholipase); VGCC (voltage gated Ca^{2+} channel).

of glutamate is coupled with postsynaptic depolarization. Accordingly, pharmacological studies have shown that this distinct form of LTP in the CA1 area is selectively prevented after the addition of competitive antagonists of the NMDA receptor site such as 2-amino-5-phosphonovaleric acid (APV).

Considerable debate has emerged about the question whether the increase in synaptic function during LTP takes place on the pre- or postsynaptic side of the synapse. Conclusive evidence has been found showing that increases in synaptic strength result from a modification in the AMPA receptor number and function at the postsynaptic side of the activated synapse. The expression of LTP was shown to be caused both by the phosphorylation of AMPA receptors and the delivery of additional receptors to the postsynaptic membrane. However, evidence also exists in support of changes in the presynaptic neuron. These data describe an increase in the probability of neurotransmitter release following the induction of LTP, which could potentially occur in concert with modifications at the postsynaptic side of the same synapse population (reviewed in Malenka and Nicoll, 1999; Lisman J, 2003).

1.4. Phases of Long-Term Potentiation

Synaptic potentiation can be divided into several temporal stages that use different mechanisms for their induction, expression, and/or maintenance. An NMDA-dependent increase in Ca^{2+} that is insufficient to generate LTP results only in the first and often second phase of synaptic potentiation, i.e. posttetanic potentiation (PTP) and short-term potentiation (STP). The induction of PTP results in enhanced potentials returning to baseline levels after 5-10 min, whereas STP lasts 60-90 min. When induced and expressed, LTP is divided into two major phases, early-LTP and late-LTP (Matthies et al., 1990). The early-phase of LTP (E-LTP) is transient, stable for up to 2-3 h, induced by

second messenger cascades activated by Ca^{2+} influx, and maintained by activated kinases (reviewed by Malenka and Nicoll, 1999; Soderling and Derkach, 2000). Late-LTP (L-LTP) begins gradually during the first 1-3 h and can last for 6-10 h in hippocampal slices in vitro or days to months in vivo (Fig. 3).

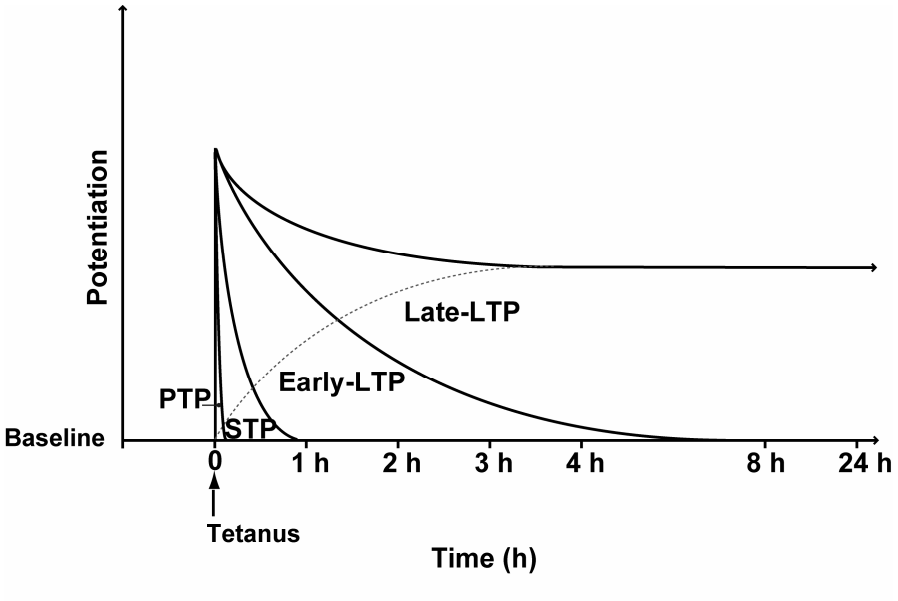

Figure 3. The multiple phases of LTP. See text for a detailed description.

The different forms of LTP can be induced in acute slices using separate stimulus protocols (Frey et al., 1993; Huang and Kandel, 1994; Nguyen et al., 1994). A single high-frequency stimulus train of distinct stimulation strength immediately induces E-LTP that lasts for up to 3 h, but is normally not sufficient to induce L-LTP. The induction of L-LTP, on the other hand, requires repeated or stronger trains of high-frequency stimulation. The activation of heterosynaptic inputs has been proposed to be required for the induction of L-LTP (Morris and Frey, 1997; Bailey et al., 2000). Stimulation of modulatory subcortical inputs (i.e., dopaminergic fibers) as well as hippocampal excitatory synapses has been shown to induce and maintain L-LTP in area CA1 (Frey et

al., 1990; Frey et al., 1991; Huang and Kandel, 1995; Swanson-Park et al., 1999). Considerable research is currently underway to investigate the cellular mechanisms including the nature of plasticity-related proteins involved in L-LTP. The early and late phases of LTP involve different signaling pathways. In contrast to E-LTP, L-LTP requires PKA-activity pathways (Frey et al., 1993; Huang and Kandel, 1994; Abel et al., 1997; Nyugen and Kandel, 1997). Furthermore, L-LTP is distinguished from E-LTP by the requirement for protein synthesis (Krug et al., 1984; Frey et al., 1988; Huang and Kandel, 1994; Nguyen et al., 1994; Frey et al., 1996; Huang et al., 1996). By using suppressors of RNA-translation, studies have shown that late stages of LTP (> 3 h) require protein expression (Krug et al., 1984; Stanton et al., 1984; Deadwyler et al., 1997; Frey et al., 1988; Frey et al., 1996; Mochida et al., 2001). Protein synthesis is assumed to be necessary for the cell to maintain synaptic changes over long time periods, which requires constant molecular turnover and eventually leads to synaptic growth. It is hypothesized that L-LTP requires the activation of transcription factors for sustaining long periods of synaptic enhancement and finally making the synaptic change permanent. In support of this theory it has been shown that the transcription factor cAMP-responsive element binding protein (CREB) differs in its activation following the induction of either the short or long form of LTP (Matthies et al., 1997; Impey et al., 1998; Schulz et al., 1999; but see Gass et al., 1998; Balschun et al., 2003). Only L-LTP is accompanied by sustained CREB phosphorylation (Schulz et al., 1999), or CREB-mediated transcription (Bito et al., 1996; Impey et al., 1996; Impey et al., 1998; Deisseroth and Tsien, 2002).

The theory that the activation of nuclear transcription factors are required for L-LTP raises the problem of synaptic input specificity. The question arises how proteins produced in the soma or dendritic compartments are targeted to LTP-expressing synaptic inputs rather than traveling to any synapse within the cell. It has been proposed

that the synapse produces a local marker after an appropriate stimulus event that allows it to sequester proteins in response to nuclear signals (Frey and Morris, 1997). However, the proteins and possible markers involved in such a mechanism have not yet been identified experimentally.

1.5. CREB: a Possible Modulator of Long-Term Plasticity

De novo gene expression and protein synthesis, which are initiated by the activation of transcription factors, are required to maintain plastic changes for long periods after learning (Davis and Squire, 1984; Matthies, 1989; Izquierdo et al., 1997; Schafe et al., 1999; Vianna et al., 2001; Igaz et al., 2002) as well as for the induction of L-LTP (Krug et al., 1984; Frey et al., 1988; Nguyen et al., 1994, Kandel and Pittenger, 1999). However, the cellular mechanisms that initiate gene expression subsequent to long-lasting enhancement of synaptic transmission are uncertain. Activation of single transcription factors by multiple pathways makes them of particular interest as candidate mechanisms responsible for modulating long-term plasticity changes. The activation of a single transcription factor CREB by multiple signaling pathways known to be involved in memory as well as LTP has created great interest into CREB activation as a candidate mechanism in the long-term modulation of synaptic change. In addition, as one of the proteins that may be activated during L-LTP, CREB has been studied particularly based on the suggestion that this transcription factor may play a significant role in memory formation (reviewed in Frank and Greenberg, 1994; Silva et al., 1998; Deisseroth et al., 2003).

CREB is a member of the basic leucine zipper superfamily of transcription factors that modulates the transcription of genes by binding to a regulatory DNA promoter known as cAMP responsive element (CRE) (Brindle and Montminy, 1992; Mayr and Montminy,

2001). Nuclear CREB can be activated by several neural signaling pathways, including the cAMP and Ca^{2+} pathways which are known to be involved in memory and are activated or up-regulated by stimuli that induce LTP (see West et al., 2001; Deisseroth et al., 2003). A variety of kinases induced by these pathways have been shown to both activate CREB by phosphorylating the Ser 133 site (Gonzalez et al, 1989; Sheng et al., 1991; Bito et al., 1996; Impey et al., 1998; Deisseroth and Tsien, 2002; Ying et al., 2002), as well as play critical roles in the induction and/or maintenance of LTP (Silva et al., 1992; Frey et al., 1993; Huang et al., 1994; Patterson et al., 1996; Impey et al., 1998; Nicoll, 2003). These kinases include protein kinase A (PKA), protein kinase C (PKC), as well as the calmodulin kinases (CamKs). The activation of CREB by phosphorylation at the Ser 133 site causes CREB to become active in promoting transcription from the CRE (Dash et al., 1991; Hunter and Karin, 1992; Chrivia et al., 1993; Mayr and Montminy, 2001; see Fig. 2). CREs lie upstream from a number of genes, including immediate early genes such as *c-fos* (Sassone-Corsi et al., 1988) and *zif/268* (Sakamoto et al., 1991), which have been shown to be required in L-LTP or to be selectively expressed in association with neuronal activity and subsequent CREB activation (Morgan and Curran, 1989; Dash et al., 1991; Scheng et a., 1991; Worley et al., 1993; Alberini et al., 1995; Huang et al., 1996; Guzowski et al., 2000; Jones et al., 2001; Ying et al., 2002; Bozon et al., 2003). Immediate early genes activated by neuronal activity could in turn transcribe late-effector genes that encode the proteins underlying structural changes at the synapse, which are believed to be the final result of long-term plasticity changes. The activation of CREB by converging signal pathways induced by multiple synaptic inputs could be a mechanism that controls the consolidation of lasting forms of synaptic plasticity, such as L-LTP. Research has focused on understanding the relationship between CREB activation and LTP due to the considerable overlap in their signaling mechanisms.

Considerable evidence has implicated CREB in the formation of memory (reviewed in Frank and Greenberg, 1994; Silva et al., 1998) particularly in invertebrate models, such as *Aplysia* and *Drosophila*. In the mollusc *Aplysia*, long-term facilitation is blocked by the injection of CRE oligonucleotides into the presynaptic nucleus (Dash et al., 1990), and in *Drosophila*, long-term memory disappears or is increased after the repression or the induction of activated forms of CREB (Tully et al., 1994; Yin et al., 1994, 1995). However, in the vertebrate hippocampus, the specific role of CREB in memory has not been clear, perhaps due to the complexity of signaling pathways in the mammalian nervous system. In mutant mice, where $CREB^{\alpha\Delta}$ was inactivated by homologous recombination, animals displayed deficits in long-term memory, including spatial memory (Bourtchuladze et al., 1994; Kogan et al., 1996). Another group verified that CREB deficient mutant mice had deficits in long-term memory, but also provided evidence that this deficit was not related to hippocampal-dependent spatial memory (Gass et al., 1998). Mutant mouse strains that have a progressively more complete deficiency of all CREB isoforms, also showed only subtle effects on hippocampal-dependent types of learning and memory (Balchun et al., 2003) suggesting that perhaps CREB activation is a covariate of general hippocampal activity rather then a critical modulator of hippocampal types of learning and memory or is compensated for by other transcription factors in these genetically modified mice. However, due to the central role of CREB in development, general neuronal activity deficiencies caused by deregulation in mutant mice could lead to memory abnormalities. Therefore, it is important to test whether acute modulations of CREB function also affect memory, as well as to determine the direct role of CREB in plasticity models for memory formation such as LTP.

Electrophysiological data support a functional role for CREB in hippocampal LTP. The activation of CREB is specific for LTP-inducing stimuli, both events share common signaling mechanisms, and the dynamics of both processes are regulated through the

temporal patterns of synaptic input. It was initially of particular significance to investigate the physiology of CREB activation and to establish that the activation of CREB is correlated with the induction of LTP. CREB activation can be detected in hippocampal neurons using an antibody specific to phosphorylated CREB (at the Ser 133 site; Gity et al., 1993). Many studies have used this approach to observe the activation of CREB in response to electrical stimulation. By using this technique it was found that CREB phosphorylation was not just a general marker for neuronal activity, but was rather specifically activated in response to synaptic stimulation (Deisseroth et al., 1996). Only tetani that generated robust, long-lasting potentiation (18 sec at 50 Hz) activated CREB in immature disassociated hippocampal neurons (Deisseroth et al., 1996). However, Ser 133 phosphorylation of CREB alone did not necessarily result in CREB-dependent gene expression (Bito et al., 1996). Only longer synaptic stimulation (180 sec at 5 Hz, rather than 8 sec) resulted in a sustained phosphorylation of CREB that was accompanied with CREB-dependent gene expression (Bito et al., 1996). These findings in immature hippocampal-neuron cultures were confirmed in acute slices from transgenic mice. Transgenic mice with a CRE-regulated reporter construct revealed that CRE-mediated gene expression is increased in response to stimuli that generate L-LTP (Impey et al., 1996). The induction of E-LTP with a brief stimulus (1s at 100 Hz) phosphorylated CREB, but failed to induce CREB-dependent transcription. Tetani given at a higher frequency (3 tetani at 5 min intervals) resulted in both protein-synthesis dependent L-LTP (4 h) and CRE-mediated gene expression (Impey et al., 1996). Therefore, it was shown that both phases of LTP are associated with the phosphorylation of CREB, but that only the activation of L-LTP by repeated tetanic stimulation, which induces protein synthesis dependent LTP (Frey et al., 1993; Huang and Kandel, 1994; Nguyen et al., 1994), resulted in CREB-mediated transcription (Impey et al., 1996), which is assumed to be responsible for long-term plasticity. These findings

naturally led to the investigation of the temporal dynamics of CREB activation during both E-LTP and L-LTP.

Matthies et al., (1997) investigated the temporal dynamics of CREB activation in acute hippocampal slices and observed that high-frequency stimulation (100 Hz) of the Schaffer collaterals resulted in L-LTP (4 h) associated with an immediate increase in CREB phosphorylation apparent in protein fractions of area CA1 as well as by immunohistochemistry. Fluorescent imaging of the entire hippocampal CA1 region revealed an increase in CREB phosphorylation after the induction of LTP, but this increase was transient and appeared to decline after 30 min despite continued synaptic enhancement. The temporal dynamics of CREB phosphorylation during hippocampal LTP in vivo were shown to be biphasic, and depended on the type of LTP induced. L-LTP induced in the perforant path in vivo (200 Hz) appeared to result in an immediate increase in CREB phosphorylation at 30 min followed by an additional long-lasting peak at 2 h. This later sustained phosphorylation of CREB was shown to last for up to 24 h, whereas E-LTP resulted in transient CREB phosphorylation that disappeared by 2 h, as shown by optical imaging of the entire population of dentate granule cells (Schulz et al., 1999). It was shown that sustained CREB phosphorylation was invariably generated by stimuli that also induced L-LTP similar to the findings in cultured immature hippocampal neurons (Bito et al., 1996). Although the evidence presented in vivo suggests a possible correlation between the sustained phosphorylation of CREB and the maintenance of L-LTP, the temporal dynamics of CREB phosphorylation in vivo clearly differ from those observed in acute hippocampal slices (Matthies et al., 1997), bringing into question whether acute hippocampal slices are an appropriate model in the investigation of cellular CREB activation. In addition, both studies inferred an increase in CREB phosphorylation by visually comparing fluorescent images of entire neuronal populations to control images. An actual quantification of nuclear CREB phosphorylation at a single-

cell resolution during the multiple phases of LTP could more conclusively reveal the dynamics of CREB activation during hippocampal long-term synaptic plasticity. Revealing the temporal relationship among these events is important in elucidating their possible causal relationship.

Electrophysiological investigation of the role of CREB in LTP using CREB mutant mice has also produced conflicting results. Acute hippocampal slices from $CREB^{\alpha\Delta}$ mutant mice were severely impaired in the maintenance of LTP lasting longer than 2 h after strong tetanization (100 Hz) of the Schaffer collateral fibers, however, E-LTP was not affected (Bourtchuladze et al., 1994). However, these results have not been reproduced (personal communication, JU Frey), and additional data have contradicted their results. L-LTP in acute hippocampal slices from mice with a progressive reduction of CREB in the CA1 area as well as from mutants completely lacking CREB in the entire brain was not affected (Gass et al., 1998; Balschun et al., 2003), suggesting that CREB may not play a pivotal role in hippocampal-dependent synaptic plasticity, a view supported by previous findings that suggest CREB plays different roles according to the mode of LTP induction (Pittenger et al., 2002).

The fact that the temporal dynamics of CREB activation are related to the phases of LTP has lead to the investigation of the signaling cascades that mediate CREB phosphorylation. The significance of multiple pathways signaling CREB activation could serve to carry information to the nucleus about a specific set of synaptic stimuli. Electrical stimulation of mouse dorsal root ganglion neurons in culture showed that the temporal dynamics of intracellular signaling patterns are important for ensuring the pattern of gene expression. The temporal features of action potentials and Ca^{2+} transients were shown to regulate the expression of the immediate early gene *c-fos* (Fields et al., 1997). Only stimuli with the appropriate temporal patterns to induce the ERK/MAPK pathway in concert with CREB phosphorylation resulted in expression of *c-*

fos. Impey et al. (1998) further showed that sustained CREB phosphorylation in cultured hippocampal neurons is dependent on the activation of the extracellular signal-related protein kinase (ERK)/MAPK pathway, with the persistent activation of ERK increasing CRE-mediated gene expression. This transcription also required the activation of the PKA pathway. PKA is required for the translocation of ERK to the nucleus for the activation of CREB kinase Rsk2 (see Fig. 2). These results show that the temporal patterns of synaptic input also modulate CREB-dependent transcription through the activation of distinct kinases.

Further investigation of activity-dependent CREB phosphorylation has shown, by direct depolarization of hippocampal neurons in vitro, that synaptic stimulation recruits first the fast CaMK pathway responsible for early CREB signaling, followed by a slower MAPK pathway shown to mediate long-term CREB phosphorylation (Wu et al., 2001). Short Ca^{2+} signals produced rapid CaMK signaling (Finkbeiner et al., 1997) such as stimuli that induce E-LTP, whereas stronger intracellular Ca^{2+} transients produced both rapid CaMK and slow MAPK signaling. The coupling of the two pathways resulted in sustained CREB phosphorylation. These findings suggest that the kinetics of intracellular signaling pathways can each in turn convey information to the nucleus about synaptic stimuli, such as their timing or duration. Such research could lead to evidence that would support the idea that CREB is part of an important switch that translates short-term into long-term plasticity after appropriate stimulation, and provide insight into whether CREB activation is required for LTP or one of its phases.

1.6. The Study of LTP in Hippocampal Slices

If in fact the protein synthesis dependent late phases of LTP are required for memory formation by causing long-lasting changes in synaptic strength and structure, then

understanding the mechanisms and events by which this occurs is extremely important, but technically difficult. Investigating the mechanisms of LTP has been greatly aided by the use of acute hippocampal slices (Schwartzkroin and Wester, 1975). Their easy access to each of the components of the hippocampal trisynaptic cascade has made them one of the most widely studied models for synaptic plasticity in vitro (Bliss and Collingridge, 1993; Bliss et al., 2003). The hippocampal brain slice retains its intrinsic circuitry, along with the major hippocampal cell types within a 400 μm transverse slice (see Fig. 1b), and a majority of LTP research to date has been done using acute brain slices that remain viable for several hours. Extending the viability of acute slices has resulted in important advances in understanding the protein-synthesis dependent phase of LTP. However, their viability remains relatively short compared to behavioral models of learning and memory, limiting the use of acute slices for investigating the mechanisms involved in the maintenance of long-term LTP. To study the full time course of hippocampal LTP typically requires in vivo studies, which are confined by limited accessibility to the hippocampus, a lesser degree of experimental control, and limited to extracellular field-potential recordings.

Imaging experiments with fluorescent labeling can observe the proteins involved in L-LTP in hippocampal neurons. However, the associated methods can last days and require the use of cultured hippocampal tissue, such as dissociated cell cultures or organotypic slices. Cultured slices may prove to be of advantage in monitoring live biochemical changes in parallel to changes in synaptic strength with appropriate spatiotemporal resolution. LTP research using cultured hippocampal neurons and organotypic slices have allowed scientists to overcome the time restraint of acute hippocampal slices as well as provide experimental freedom that is not possible in vivo.

Hippocampal slice cultures can be maintained for weeks with viable connections, making them an ideal model for experiments that require extensive manipulation or time

(Gähwiler 1981, Gähwiler et al., 1997; Stoppini et al., 1991; Evans et al., 1998). However, due to the difficulty in which adult tissue can be cultured, such LTP research has been limited to the study of embryonic, perinatal, and juvenile cultured slices, which develop their cytoarchitecture in vitro (Zhabotinski et al., 1979; Buchs et al., 1993; Müller et al., 1993; Sakaguchi et al., 1994). Their neurons do not acquire the same anatomical and functional maturity as neurons that have matured in vivo. In vitro studies of synaptic plasticity for long periods and when experiments require many days of incubation such as for optical imaging and protein labeling are thus limited to cultures of initially immature hippocampal synapses. For the study of LTP, and particularly its late phases, this is limiting as recent evidence has shown that the mechanisms for synaptic plasticity in neonatal or juvenile brains differs from those in more mature, functioning neural circuits (Yasuda et al., 2003).

Immature hippocampal cultures do not only differ in the anatomical organization and maturity of their neurons, but also in the mechanisms underlying LTP. It was initially thought that LTP in general only occurred in the well established mature hippocampal synapse, as it was not possible to induce LTP with tetanic stimulation in slices from juvenile rats (< postnatal day 10; P10)(Baudry et al., 1981; Harris et al., 1984; Bekenstein and Lothman, 1991; Dudek and Bear, 1993; and Bolshakov and Siegelbaum, 1994). However, it was found that a distinct form of LTP could be induced in hippocampal slices from neonatal animals (< 8 days old) by using a stimulation protocol that pairs presynaptic stimulation with postsynaptic depolarization (Durand et al., 1996; Liao et al., 1996). The failure of immature hippocampal neurons to respond to tetanic stimuli was shown to be a result of the lack of functional AMPA receptors at the young synapse, shown by an absence of synaptic current at resting membrane potential despite presynaptic glutamate release (Durand et al., 1996; Liao et al., 1996). A pairing protocol caused these ‘silent synapses’ (i.e., those composed of only NMDA receptors)

to recruit functional AMPA receptors and increased synaptic currents (Isaac et al., 1995; Liao et al., 1995; Liao et al., 1996; Durand et al., 1996). The percentage of silent synapses decreases during the first postnatal weeks of hippocampal development, but they continue to be present during the second and third postnatal week (Isaac et al., 1995; Liao et al., 1995). These findings suggest that the basic mechanism of LTP in young rats (< P10-12) is based on a transformation of silent synapses into functional ones by recruiting additional AMPA receptors. Studies in thalamocortical synapses add to the evidence that postsynaptic modification in AMPA receptor function underlies LTP at immature synapses, where synapses with low AMPA/NMDA ratios convert to higher AMPA/NMDA ratios during LTP (Isaac et al., 1997).

There are additional differences at young compared to mature hippocampal synapses with respect to synaptic transmission. NMDA receptor channels in young neurons have longer open times, differences in the voltage-dependent Mg^{2+} block prior to P20 (Monyer et al., 1994; Kutsuwada et al., 1996; Takahashi et al., 1996), a higher affinity for glutamate (Kutsuwada et al., 1996), lower levels of CaMKII (Kelly and Vernon, 1985), and a higher affinity for Ca^{2+}/calmodulin(CaM)-dependent adenyl cyclase (Constantine-Paton and Cline, 1998). In addition, the glutamate transporters responsible for buffering glutamate in the synaptic cleft are expressed late into the second postnatal week (Diamond et al., 1997; Ullensvang et al., 1997). Even though the CA1 pyramidal neurons of the rat hippocampus have fully completed the expansion of their apical dendrites into the dendritic layer, stratum radiatum, by P1 (Bayer et al., 1980), it is clear that the functional molecular components of their synapses are still developing. Prior to P12 half of the synapses in the CA1 region occur on dendritic shafts rather then spines (Fiala et al., 1998), a possible reason for the lack of long-term LTP in immature hippocampal neurons for dendritic spines provide synapse specificity and biochemical departmentalization.

Current findings show that synaptic plasticity is dependent on different signaling cascades during development. It was shown by Yasuda et al. (2003), that the induction of LTP in the CA1 region of mature rats (> P 20) required CaMKII, whereas LTP in the neonatal hippocampus (< P9) required PKA. In addition, CaMKII and MAPK are both required for the expression of LTP (Malenka et al., 1999; Lisman et al., 2002; Yasuda et al., 2003), whereas PKA is only required for its long-term maintenance at the mature synapse (Frey et al., 1993; Huang and Kandel, 1994; Abel et al., 1997; Nyugen and Kandel, 1997). In contrast, LTP in the immature hippocampus does not require CaMKII or MAPK activity, but is solely dependent on PKA activity for its induction, in part by modulating synaptic AMPA receptor activity (Yasuda et al., 2003). These kinases all participate in the activation of nuclear transcription factors, such as CREB, in response to synaptic input resulting in LTP (Impey et al., 1998; Wu et al., 2001; Deisseroth and Tsien, 2002) suggesting that their activation in immature systems of synaptic plasticity may not correspond to those in mature neural circuits.

The facts that cultured slices are necessary for long-term investigations, and considering that adult tissue rarely survives in culture has led to the common use of juvenile hippocampal slices from P10 animals in LTP investigations. Synaptic transmission can be detected at resting membrane potentials as in adults (Hestrin et al., 1990), many gross developmental processes have been completed, and tissue from P10 animals can readily be maintained in culture in contrast to tissue from more mature animals. However, current findings that synaptic plasticity is dependent on different signaling cascades during development (Yasuda et al., 2003), causes hesitation in comparing data from P10 animals to those in mature systems.

Mature hippocampal cultures could overcome the experimental limitations of previous culture models in the study of LTP, and a method for the long-term maintenance of mature hippocampal slices in vitro has recently been introduced. Xiang et al. (2000)

demonstrated that cultured mature slices (P20-30) remained viable, morphologically intact, and showed field potentials after several weeks in culture. They proposed that neuronal plasticity of the adult hippocampus could be studied in their slice cultures after neurogenesis and synaptogenesis has been completed in vivo. However, it has not been determined whether cultured mature slices maintain normal synaptic function and recording stability to provide a model system for the study of LTP and, in particular, its late phases.

1.7. Aims of the Dissertation

This study investigated whether mature cultured hippocampal slices could be used for long-term electrophysiological experiments and particularly for the study of long-term changes in synaptic plasticity, which is not feasible in acute slices for prolonged periods of time or of limited relevance in the immature slice due to the differences in the cellular processes of synaptic plasticity at the immature synapse. Mature cultured hippocampal slices were characterized using electrophysiological and immunohistochemical techniques to determine whether mature hippocampal tissue can retain normal synaptic function and recording stability after extended time in culture. Data is presented describing the effects of culture processes on hippocampal structure and function, as well as the development of adapted methodological procedures required to obtain and sustain stable electrophysiological properties in mature hippocampal-entorhinal cortex slices (P25-30) for use in LTP experiments. The electrophysiological properties of mature cultured slices were assessed, in particular the induction and maintenance of long-lasting LTP. In addition, I present data describing the extracellular stimulation requirements for LTP induction and its maintenance for > 4 h at the Schaffer-collateral-CA1 synapse, and determine the type of LTP expressed in these slices. After

establishing protocols for the use of mature hippocampal cultures in electrophysiological experiments, and characterizing the properties of LTP they express, mature cultured hippocampal-entorhinal cortex slices were continually used throughout this dissertation as the model in the study of long-term plasticity changes.

Mature hippocampal-entorhinal cortex slices were used in this study to determine the relationship between the maintenance of long-lasting LTP and CREB activation. It is hypothesized that sustained activation of the nuclear transcription factor CREB is needed to maintain long-term LTP. The hypothesis was tested in mature hippocampal-entorhinal cortex slices (P25-30) by quantifying changes in CREB phosphorylation for individual CA1-pyramidal neurons during the maintenance of L-LTP using confocal microscopy. Measuring the extent of CREB phosphorylation at a single-cell resolution in mature intact hippocampal tissue during the maintenance of LTP revealed the dynamics and extent of CREB phosphorylation for individual neurons undergoing long-lasting changes in synaptic strength. The findings in this dissertation characterize LTP in cultured mature slices in order to validate their use for further experiments investigating the specific mechanisms involved in LTP, specifically the role of the nuclear transcription factor CREB in the maintenance of long-lasting LTP.

2. Methods

2.1. General Methods

2.1.1. Mature Hippocampal-Entorhinal Cortex Slice Preparation

Organotypic hippocampal slice cultures were prepared from 25-30 day old male Wistar rats (SHOE, Institute breeding stock, Magdeburg, Germany) by using methods that were modified from those described previously (Xiang et al., 2000). All modifications were aimed at obtaining slices that exhibited stable electrophysiological signals and reliable long-term potentiation. Rats were calmed with a brief exposure to halothane and anaesthetized using 0.18 g/kg ketamine hydrochloride (Sigma), which is the required dose for maximum neuronal protection (Lees, 1995). The animals were then left to rest for 6 min in a chamber filled with carbogen (95 % O_2/5 % CO_2), before decapitating them and removing their brains. The hemispheres were separated mid-sagittally and cooled in ice-cold modified Gey's balanced salt solution (mGBSS) saturated with carbogen for 10 min. The mGBSS was composed of 1.5 mM $CaCl_2$, 4.9 mM KCl, 0.2 mM KH_2PO_4, 11 mM $MgCl_2$, 0.3 mM $MgSO_4$, 130 mM NaCl, 2.7 mM $NaHCO_3$, 0.8 mM $NaHPO_4$, 22 mM NaHEPES, and 5 mM glucose, pH 7.32. A scalpel was used to make a 50-70 ° cut along the dorsal edge of each hemisphere laying flat on its medial surface (see Fig. 4). A hemisphere was then glued (Histoacryl, Braun) to a Teflon platform, surrounded by frozen mGBSS, oriented with the freshly cut surface facing down. The tissue was submerged in chilled mGBSS and 400 μm thick sections were cut from posterior to anterior using a vibratome (Cambden, UK) modified to minimize Z-axis oscillation. Vibratome sections were taken perpendicular to the septo-temporal hippocampal axis. The hippocampal formation, subicular and entorhinal cortices, as well as the cortices dorsolaterally adjacent to hippocampus were taken from the entire 400 μm section. Only hippocampal slices with cell layers that appeared transparent and intact were placed on

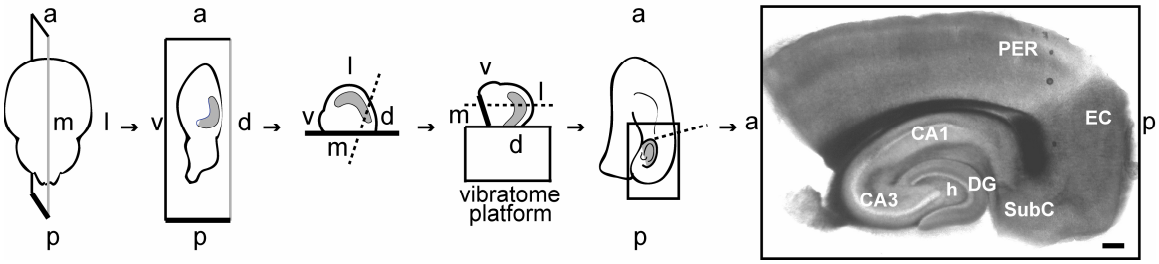

Figure 4. Schematic of the sequential steps in the preparation of hippocampal-entorhinal cortex slices from young adult rats (P25-30). Dashed lines indicate the orientation and angle of each cut and the hippocampal formation is represented (*grey*) throughout the procedure. The brains were first cut mid-sagittally and at a 50-70° angle along the dorsal edge of each hemisphere and were then mounted on the vibratome platform to be sectioned perpendicular to the septo-temporal hippocampal axis. Vibratome sections were taken from each hemisphere oriented with the newly cut surface down. The hippocampal formation, subicular and entorhinal cortices, as well as the cortices dorsolaterally adjacent to hippocampus were taken from the entire 400 μm section (*small inset*). A representative transmitted light image of a freshly prepared hippocampal-entorhinal cortex slice is shown (*right*). Scale bar: 400 μm. a, anterior; p, posterior; m, medial; l, lateral; d, dorsal; v, ventral; PER, perirhinal cortex; EC, entorhinal cortex; SubC, subicular cortices; DG, dentate gyrus; h, hilus.

25 mm culture membrane inserts (NUNC, 0.2 μm anapore membrane) in a 6-well cluster dish (Sigma) with 1 ml of high K^+ culture media (25 % heat-inactivated horse serum, Lot-Nr.: 3042915D, GIBCO-Life Technologies; 40 % Basal Essential Media, Eagle's (BME), Sigma; 25 % Earle's Balanced Salt Solution (EBSS), Sigma; 10 % 250 mM NaHepes in BME, (pH 7.3); 0.5 mM L-glutamine, Sigma; 28 mM glucose, pH 7.32). The slices were incubated overnight at 34 °C in a humidified carbogen atmosphere (95 % O_2/5 % CO_2), and then transferred to ambient O_2 and 5 % CO_2 the following morning. After 3 days the slices were switched to media with decreased K^+ levels, decreased horse serum concentration, and increased L-glutamine levels (5 % heat-inactivated horse serum, 20 % BME, 65 % modified Earle's Balanced Salt Solution (mEBSS), 10 % 250 mM NaHepes in BME, (pH 7.3); 1.0 mM L-glutamine, 28 mM glucose, pH 7.32). The mEBSS was self-made and composed of 1.8 mM $CaCl_2$, 2.0 mM $MgSO_4$, 52.5 mM NaCl, 30.9 mM $NaHCO_3$, 0.9 mM NaH_2PO_4, prepared in sterile H_2O (GIBCO-Life

Technologies), pH 7.32. Formulating the mEBSS without K^+ salts resulted in a final K^+ concentration of 1.6 mM for the tissue culture media. Testing several lots of horse serum revealed considerable differences in the survival of the neuronal circuitry and the reliability of LTP induction. Serum from a lot that resulted in low levels of reactive gliosis and promoted neuronal survival was used throughout all LTP experiments. The culture medium was exchanged every 3 days. For experiments investigating cellular CREB phosphorylation two slices were always cultured on one membrane to provide a control slice for electrophysiological and immunohistochemical experiments.

2.1.2. Electrophysiology

Following cultivation for 10-15 days, slice cultures were transferred to a carbogen-interface recording chamber (Scientific Systems Design, Inc.) that was modified to fit tissue culture inserts and optimized for long-term recordings of cultured slices using enhanced humidification (≥ 90% saturation). Slice cultures were superfused with artificial cerebrospinal fluid (ACSF; 2.5 mM $CaCl_2$, 20 mM glucose, 1,0 mM L-glutamine, 3 mM KCL, 1.24 mM KH_2PO_4, 110 mM NaCl, 25.6 mM $NaHCO_3$, 1.5 mM $MgSO_4$, osmolarity adjusted to the level of the culture media) saturated with carbogen at 32 °C.

Extracellular field excitatory postsynaptic potentials (fEPSPs) and/or population spikes were recorded after stimulation was applied at constant current (A385 Stimulus Isolator; WPI) using a stainless-steel stimulation electrode (AM-5710, 5 MΩ, Zak-Medizin Technik) altered to result in a final tip resistance of 400-800 kΩ. Signals were amplified by the Axonclamp 2B amplifier (Axon Instruments), digitized using a CED 1401 A/D converter, and analyzed with custom-made software (PWIN, Magdeburg, Germany).

LTP induction. Mature hippocampal slices (10-15 DIV) were allowed to rest in the recording chamber for 30 min and a stainless-steel stimulation electrode (Zak-Medizin Technik) was then positioned in the stratum radiatum of the CA1 region for stimulation.

FEPSPs were recorded extracellularly in the CA1 region of the stratum radiatum with a glass capillary microelectrode filled with ACSF (17 μm; resistance 400-800 kΩ), and the slope (mV/ms) of the fEPSP was measured. Stimulation was applied at constant current with the stimulation intensity adjusted to result in 50 % of the maximum fEPSP and held constant throughout the experiment. Once a baseline was recorded for 1 h a specified tetanization protocol was used to induce LTP. After the tetanus, recordings were taken at 1 min and every 5 min thereafter. The average slope of the baseline recordings was compared to the slopes after tetanization using the two-tailed Mann-Whitney *U*-test ($P<0.05$). The fEPSP amplitudes and slopes are reported as the mean ± SEM.

2.1.3. Immunohistochemistry

The hippocampal-entorhinal slice cultures were fixed in 2.5 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4) for 30 min at 4 °C. Fixated slices were then removed from the anapore membrane and cryoprotected in 30 % sucrose in PBS overnight at 4 °C. Forty-five μm sections were cut using a cryostat and the free-floating sections were washed 2 times in chilled PBS. The sections were rocked for 2 h at room temperature in ROTI *Immunoblock* (Roth GmBH, Germany) diluted 1:10 in PBS with 0.5 % Triton X-100, and rocked overnight at 4 °C with primary antibodies diluted in blocking solution. Primary antibodies were as follows: anti-ß-III-tubulin (monoclonal IgG_2b, 1:500; Sigma), anti-chondroitin sulfate proteoglycans (CSPGs, polyclonal, 1:1000; Sigma), anti-CREB (monoclonal IgG_1, 1:300; Zymed Lab. Inc.), anti-glial fibrillary acidic protein (GFAP, polyclonal, 1:1000; Sigma), anti-glutamic acid decarboxylase (GAD 67, polyclonal, 1:1000; Chemicon), anti-MAP2a,b (polyclonal, 1:500, Sigma), anti-neurofilament 68 (monoclonal IgG, 1:1000; Transduction Laboratories), and anti-phosphorylated CREB (polyclonal, 1:300; New England Biolabs). After incubation, the slices were returned to room temperature and rocked for 2 h, then rinsed 3 times in PBS.

All secondary antibodies (Molecular Probes) were conjugated with Alexa dyes and diluted to a working solution of 1:200. Following 2 h incubation at room temperature, slices were washed 3 times in PBS and mounted on glass coverslips using anti-fade (Molecular Probes) to slow fluorescent decay. Specimens were examined using a Leica CLSM (TCS-SpectroPhotometer 1; Heidelberg, Germany) confocal laser-scanning microscope. Immunofluorescence images were obtained as Z-series stacks and analyzed for localization of staining using Leica imaging software.

2.1.4. Immunoblotting

Anti-neurofilament 68 antibody staining was used to indicate improved tissue culture quality by measuring the relative portion of neural protein within slices cultured for 14 DIV. For the first 3 days slices were cultured in 25 % serum. On the 3rd DIV, they were switched to low K^+ media with serum concentrations of 5 %, 25 %, or 40 % (n=12 for each group). Four slices per serum concentration group were pooled and loaded on each of 3 immunoblots. After 14 DIV the slice cultures were frozen, pooled, and then homogenized in ice-cold buffer (10 mM HEPES pH 7.9, 10 mM KCl, 10 mM EDTA, DTT, IGEPAL and one protease inhibitor cocktail tablet; Roche Diagnostic GmbH, Germany). Following homogenization, protein concentrations were measured using a Bradford (1976) analysis with bovine serum albumin as the standard. Equal amounts of protein were resolved in 12.5 % SDS-polyacrylamide gels (SDS-PAGE) and transferred to membranes for immunoblotting. Membranes were blocked in *ROTIblock* (Roth GmBH, Germany) diluted 1:10 in 1 M PBS (pH 7.4) with 0.5 % Tween (2 h) and then probed with mouse monoclonal anti-neurofilament 68 antibodies (1:1000, Transduction Laboratories). The blots were incubated in the blocking solution containing the anti-neurofilament 68 antibody overnight at 4 °C, after which they were washed three times in 1 M PBS (pH 7.4). Blots were then incubated with horseradish peroxidase-linked goat

anti-mouse IgG antibodies (2 h; 1:20,000 dilution, Amersham) before being developed using enzyme-linked chemiluminescence (ECL, Amersham).

Anti-neurofilament 68 antibody staining was also used to indicate the relative density of neurons and their processes within slices preincubated in a carbogen atmosphere. After tissue preparation, slices were incubated in carbogen for various lengths of time (0, 4 h, 8 h, and 16 h). Four slices per time point were pooled and loaded on one immunoblot. After 10 DIV slices were processed for the analysis of protein fractions as described above.

2.2. Experimental Design

2.2.1. Characterization of Mature Cultured Hippocampal-Entorhinal Cortex Slices

To determine the connectivity of the hippocampal circuit, the molecular cell layer of the dentate gyrus was stimulated using a twisted bipolar Teflon-insulated platinum electrode (17 μm; resistance 400-800 kΩ). FEPSPs and populations spikes were recorded throughout the hippocampal circuit using stainless-steel electrodes.

Analysis of electrotonic components. The contribution of electrotonic stratum oriens components to stratum radiatum fEPSPs was analyzed by placing a cut through striatum oriens fibers that project to CA1 basal dendrites. The slice was allowed to recover for 1 h, after which extracellular fEPSPs were recorded from the CA1 stratum oriens and radiatum following stimulation of the stratum oriens or radiatum. These experiments revealed potential confounds from electrotonic signals with opposite polarity, which were avoided in subsequent LTP experiments by placing the stimulation electrode closer to the recording electrode. As a standard only field potentials with a defined negative shape and apparent pair-pulse facilitation were included in subsequent experiments. In

addition, a fuchsine acid stain was performed using standard staining protocols to verify the cut placement.

Pharmacological analysis of the fEPSP: NMDA receptor contribution. Slices were preincubated in ACSF for 30 min, and baseline fEPSPs were recorded for 1 h. The following antagonists were permanently washed into the superfused ACSF in the subsequent order (n=4): a gamma aminobutyric acid A ($GABA_A$) receptor antagonist (30 μM picrotoxin, RBI), an AMPA/kainate receptor antagonist (10 μM 1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide; NBQX, RBI), and a NMDA receptor antagonist (10 μM R-(−)-3-(2-Carboxypiperazin-4-yl)propanephosphonic acid; CPP, RBI). The fEPSP amplitude after the addition of picrotoxin was considered to represent the total excitatory component of the fEPSP, composed of both AMPA and NMDA receptor currents. The AMPA receptor contribution was blocked using NBQX. The remaining component reflects the contribution of NMDA receptor currents to the fEPSP and was completely eliminated by the NMDA antagonist CPP.

LTP induction. The extracellular stimulation requirements for LTP-induction and its maintenance for > 4 h were investigated at the Schaffer collateral/CA1 synapse in cultured mature hippocampal slices using the following standard stimulation protocols: (1) Tetanization (100 Hz) consisted of 3 stimulus trains of 100 pulses at 100 Hz with a 10 min intertrain interval, later reduced to 2 stimulus trains in order to minimize after-burst discharges. (2) Tetanization (200 Hz) consisted of 2 stimulus trains of 200 pulses with a 10 min intertrain interval. (3) Theta burst stimulation consisted of 4 x 5 pulses at 100 Hz with a 250 ms interburst interval. Responses to each stimulus protocol were categorized as either potentiated (fEPSPs > 120 % of baseline responses at 1 min post tetanus), non-responsive, or resulting in after-burst discharges with subsequent depression. Potentiated slices were further classified by the duration of the potentiation (longer or less than 4 h; see Results). In addition, the fEPSP amplitudes for slices that

resulted in E-LTP were compared to those that resulted in long-lasting LTP. Only the 100 Hz tetanization protocol with 2 stimulus trains of 100 pulses at 100 Hz with a 10 min intertrain interval was used for all subsequent long-term LTP experiments.

Induction of NMDA receptor-dependent LTP. Fifty μM D,L-2-amino-5-phosphonovaleric acid (APV; Tocris) was added to the ACSF solution after a 1 h baseline recording and was perfused for 15 min before as well as during tetanization using the 100 Hz stimulation protocol. Normal ACSF was perfused for the remainder of the recording. Control LTP experiments were performed on slices from the same animal on alternating days with APV experiments, the only difference being the absence of APV (n=6). The time course of the field potential slopes was compared between control and APV treated slices using the Mann-Whitney *U*-test.

Hippocampal cell survival. For analysis of hippocampal cell survival, mouse monoclonal anti-neurofilament 68 antibody was used as a neuron-specific cell marker and rabbit polyclonal anti-glial fibrillary acidic protein as an astrocyte-specific cell marker. Images of 6 hippocampi per animal (n=4 animals) were divided between the anterior, middle, and posterior thirds of the hippocampus and analyzed for immunostaining in an area encompassing the entire granule cell layer of the dentate gyrus, the pyramidal cell layer of the CA3 region, and the pyramidal cell layer of the CA1 region. The mean cell number was determined for each group, and differences were tested for statistical significance by using the paired Student's t test. Probability values of less than 0.05 were considered significant.

Bromodeoxyuridine (BrdU) labeling. BrdU labeling was performed to determine the origins of atypical cells found in the hilus and inner molecular layer of the dentate gyrus of mature hippocampal-entorhinal cortex slices cultured in high serum concentrations (25 % serum). BrdU was used specifically to determine whether these ectopically placed neurons were dislocalized from the granule cell layer or were generated from precursor

cells in culture. BrdU is taken up only by mitotically active cells and neurons labeled by an anti-BrdU marker can be assumed to be generated from precursor cells in culture. Hippocampal-entorhinal cortex slices were first prepared from P25 rats. Part of the slices were fixated at the time of dissection, and the remaining slices were cultured in media containing 25 μm BrdU and then fixated after various times in culture. All groups were immunostained as described above with the following additional steps: free-floating sections were incubated in 50 % formamide/ 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate), followed by 2 N HCl to denature DNA. Sections were then rinsed in PBS and treated with 1 % H_2O_2 to block endogenous peroxidases (Parent et al., 1996). A mouse monoclonal anti-BrdU IgG_1 antibody (Sigma) was diluted 1:100 in blocking solution and incubated for 36 h at 4 °C. Double and triple staining was done with one of the following antibodies and analyzed for co-localization: the astrocyte specific cell-marker anti-GFAP (polyclonal, 1:1000, Sigma), the early stage neuronal marker anti-beta-III-tubulin, (monoclonal IgG_2b, 1:500, Sigma), the interneuron cell marker anti-GAD 67 (polyclonal, 1:1000, Chemicon), and the neural marker anti-neurofilament 68 (monoclonal IgG, 1:1000, Transduction Laboratories). Monoclonal antibodies used in combination for BrdU co-localization experiments were of different subtypes and tested for minimal cross-reactivity. Counting of ectopic dentate cells was performed as described above, by scoring cells with co-localized anti-beta-III-tubulin (a neural marker) and anti-BrdU (a proliferation marker).

2.2.2. Single-Cell Analysis of Nuclear CREB Phosphorylation

Confocal Microscopy and Analysis. Mature cultured slices were labeled with primary antibodies (polyclonal anti-phosphorylated CREB, New England Biolabs; monoclonal anti-CREB IgG_1, Zymed Lab. Inc.) and secondary antibodies (Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG_1, Molecular Probes).

Specimens were examined using a Leica CLSM (TCS-SP1; Heidelberg, Germany) confocal laser-scanning microscope. Confocal imaging was used to determine the ratio between phosphospecific anti-CREB (specific for Ser 133 phosphorylation of CREB, Ginty et al., 1998) and phosphorylation state-independent anti-CREB by detection of each of the labeled immunofluorescent fluorophores. Imaging protocols and settings (i.e., photomultiplier, pinhole aperture, gain, laser intensity and speed, etc.) were kept constant for all specimens examined to minimize differences between experiments. Images were recorded using 20x, and 60x oil immersion objective lenses. Only 60x images were used in quantitative CREB analysis. Imaging was performed using an Argon ion laser with two fluorescence channels that scanned the specimen at alternating intervals to eliminate non-specific autofluorescent signals. Channel one recorded emission from 488 nm excitation and channel two at 568 nm excitation. For each channel separate images were acquired, after which a composite overlay image of both channels was created using Leica imaging software. Images were collected from the neuronal cell layer of each hippocampal region (dentate gyrus, CA3, and CA1). Following image acquisition, fluorescent staining for phosphospecific anti-CREB (pCREB) and phosphorylation state-independent anti-CREB was quantified blindly, using Leica TCS-NT imaging software. By creating a circle around the circumference of a cell body, the mean fluorescent amplitude was determined from the integrated pixel intensity for each cell on both the 488 nm and 568 nm emission channels. Twenty-five adjacent cells were measured from each image.

To determine the level of CREB phosphorylation at Ser 133, a ratio of the mean fluorescent amplitude measured by the excitation of Alexa Fluor 568 bound to phosphorylated CREB was divided by the mean fluorescent amplitude measured by the excitation of Alexa Fluor 488 (CREB) for each individual cell. By creating a relative ratio of pCREB to CREB immunofluorescence inaccuracies in staining protocols were

compensated for, which allows for the comparison of phosphorylated CREB levels between slices and between experiments (modified from Carlson et al., 2000 who describe a similar method for the quantification of fluorescence DNA probes). A composite overlay image was also created using Leica imaging software to accurately portray the immunofluorescent pCREB/CREB ratio visually for each neuron. Relative pCREB/CREB ratios were determined for each cell, averaged for each experimental group, and reported as the mean ± SEM. Differences were tested for statistical significance by using the paired Student's t test. Probability values of less than 0.05 were considered significantly different.

Pharmacology. Analysis of forskolin-induced changes in CREB phosphorylation was performed on slices incubated in either the presence or absence of 20 μm forskolin (RBI Sigma). Slices were incubated in forskolin for various time periods (0, 5, 30, 60, 120, and 240 min; n=6 for each group), after which they were removed and immediately fixated for immunohistochemical investigation.

Electrophysiology. Changes in CREB phosphorylation, as indicated by changes in pCREB/CREB ratios, were assessed in slices left undisturbed in the recording chamber, slices that received continuous low-frequency stimulation (i.e., baseline tetani), as well as slices in which LTP was induced by high-frequency stimulation in the presence or absence of a competitive antagonist of the NMDA receptor site.

Control Slices. One tissue culture insert contained two slices from the same dissection. One slice was continually left undisturbed for the remainder of the experiment and a stainless-steel stimulation electrode (Zak-Medizin Technik) was positioned in the CA1-stratum radiatum of the adjacent slice for baseline stimulation. FEPSPs were recorded extracellularly in the CA1 region of the stratum radiatum with a glass capillary microelectrode filled with ACSF (tip resistance 400-800 kΩ), as described above. Following 5 h baseline stimulation, the tissue culture insert was removed and both slices

were immediately fixated for immunohistochemical investigation (n=4, for each group). Non-manipulated slices were used to determine the extent of CREB phosphorylation induced by conditions within the recording chamber. Slices receiving baseline stimulation were used to determine whether cellular activation, by stimuli not sufficient to induce LTP, caused CREB activation.

LTP Trials. Two cultured mature hippocampal slices (10-15 DIV) placed on one tissue culture insert were allowed to rest for 30 min with superfused ACSF in the optimized interface-recording chamber. Recording and stimulating electrodes were positioned on one of the slices for area CA1 recordings, whereas the adjacent slice was left undisturbed for the remainder of the experiment. Responses to Schaffer collateral stimulation in area CA1 were monitored for 1 h as mentioned above before the delivery of LTP-inducing high-frequency stimulation. High-frequency tetanization consisted of 2 stimulus trains of 100 pulses at 100 Hz with a 10 min intertrain interval. LTP was recorded for increasing time intervals (30, 120, 240 min; n=6 each group) after which electrodes were removed, and both slices were immediately fixated for immunohistochemical analysis.

CREB phosphorylation was also investigated after the induction of LTP in the presence of an NMDA receptor antagonist (APV, Tocris). Again, two slices on one insert were placed in the recording chamber and electrodes were positioned for CA1 recordings on one slice, whereas the adjacent slice was left undisturbed to serve as a control (n=6). Fifty μM APV was added to the ACSF solution after a 1 h baseline recording and was applied for 15 min before as well as during tetanization using the 100 Hz stimulation protocol. Normal ACSF was applied for the remainder of the recording (n=6). Recordings lasted 120 min following 100 Hz stimulation in the presence of APV, after which slices were immediately fixated.

3. Results and Discussion

3.1. LTP in Cultured Hippocampal-Entorhinal Cortex Slices from Young Adult (P25-30) Rats

Due to the inability to culture healthy adult tissue, many mechanistic studies of synaptic plasticity have used cultures prepared from juvenile, embryonic or neonatal brains to address questions technically limited by other models. However, the more LTP is understood in these developing neural systems, the clearer it becomes that the mechanisms involved in LTP as a process of neural formation and refinement diverges from those of mature established neural circuits. It is hypothesized that mature hippocampal cultures could overcome the experimental limitations of previous models in the study of LTP. However, it has not been determined whether cultured mature slices maintain normal synaptic function and recording stability in vitro, which is required to study LTP and in particular its late phases. This study investigated whether mature cultured slices could be used for the study of synaptic plasticity and particularly for long-term electrophysiological experiments.

3.1.1. Retention of Hippocampal Circuitry and Recording Stability in Vitro

To determine whether cultured mature hippocampal slices could serve as a model system to study LTP including its late phases, it was first determined whether these slices maintain proper synaptic connections and cellular properties to allow LTP induction in vitro. First, the connectivity between neurons in each hippocampal subregion was tested, as well as the stability of baseline recordings from neuron populations in various hippocampal regions. It was found that a previously introduced protocol (Xiang et al., 2000) often resulted in slices with damaged neurons within the CA3 or CA1 cell layers, which was apparent visually as disconnected or swollen neuronal layers when

using a dissection microscope as well as electrically after stimulation as a lack of evoked extracellular responses after 10 DIV. Using a vibratome and adding the entorhinal cortex to the hippocampal cultures resulted more reliably in slices with undamaged CA3 and CA1 pyramidal neurons. The portion of slices observed to retain undamaged cell layers increased from 38.8 ± 2.1 % to 76.4 ± 3.9 % for each dissected animal (visual observation, n=4 animals, 8 slices per animal, 4 from each hemisphere). However, regardless of the dissection protocol, the slices continued to display a progressive decrease in the amplitude and stability of extracellularly recorded field potentials with time in culture, which indicated that recovery was not reached after the initial period of cell death that was reported to last for 7 days in culture (Xiang et al., 2000).

Slices incubated overnight in a carbogen atmosphere and then transferred to ambient O_2 the following morning had healthier electrophysiological responses and a better recovery from initially low fEPSP amplitudes (at 10 DIV) in comparison to those without carbogen. Pre-incubation in carbogen enhanced slice viability by increasing neuronal survival (Fig. 5a, b). The neural density of slices incubated for various times in carbogen (0, 4, 8, and 16 h; n= 4 for each group) was assessed by immunohistochemistry and immunoblot analysis using the neuron-specific markers MAP2a,b and neurofilament 68 as indicators of neural density. The findings show that the level of neurofilament 68 protein increased in parallel to prolonged incubation in carbogen (Fig. 5c) with incubation times longer than 16 h resulting in no additional benefit (data not shown).

Slices exhibited spontaneous activity for 1 DIV to 7 DIV and were prone to after-burst discharges and oscillations within the hippocampal circuit presumably as a consequence of cell loss after the dissection. It was found that addition of the adjacent cortices to the hippocampus stabilized baseline recordings and reduced the occurrence of spontaneous activity and oscillations within the hippocampal circuit. Stable baselines and electrophysiological recordings were possible following 8 DIV and remained stable until

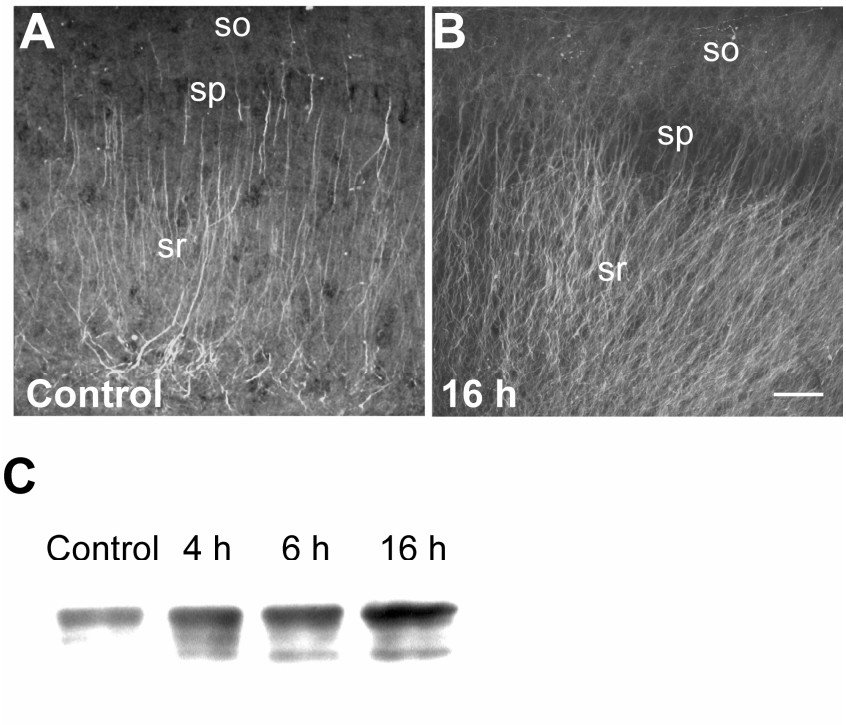

Figure 5. Carbogen pre-incubation enhanced slice viability by increasing neural survival. CA1 pyramidal cells were labeled using antibodies to MAP2a,b and visualized as composites of stacked optical sections obtained by confocal microscopy. Slices without carbogen pre-incubation (A) and slices pre-incubated in a carbogen atmosphere for 16 h (B) were subsequently cultured for 14 DIV using identical conditions. The images indicate an increase in the survival of neurons and their processes following carbogen pre-incubation, which was confirmed by using the neuron-specific marker neurofilament 68 as an indicator of neural density in immunoblots of mature slices cultured for 14 DIV. Following carbogen pre-incubation for 0, 4, 8, and 16 h (n = 4 for each group), immunostaining revealed that neurofilament 68 protein increased with longer periods of carbogen incubation (C). Scale bar (shown in *B*): 40 μm. sr, stratum radiatum; sp, stratum pyramidale; so, stratum oriens.

30 DIV (Fig. 6a). However, after 20 DIV stimulation of the CA1 stratum radiatum often resulted in late positive potentials in the recorded fEPSPs (shown in Fig. 6b) presumably due to stimulation of CA3 axon branches projecting to striatum oriens after the spreading of cell layers in response to the presence of serum in culture. Subsequent electrophysiological experiments were therefore performed between 10-15 DIV.

The fEPSP recorded in the CA1 stratum radiatum after stimulation of the Schaffer collateral fibers consisted of an earlier negative and a later positive component. The positive component of the recorded fEPSP in the CA1 stratum radiatum after stimulation of the Schaffer collaterals was thought to occur by stimulating axon branches that terminate

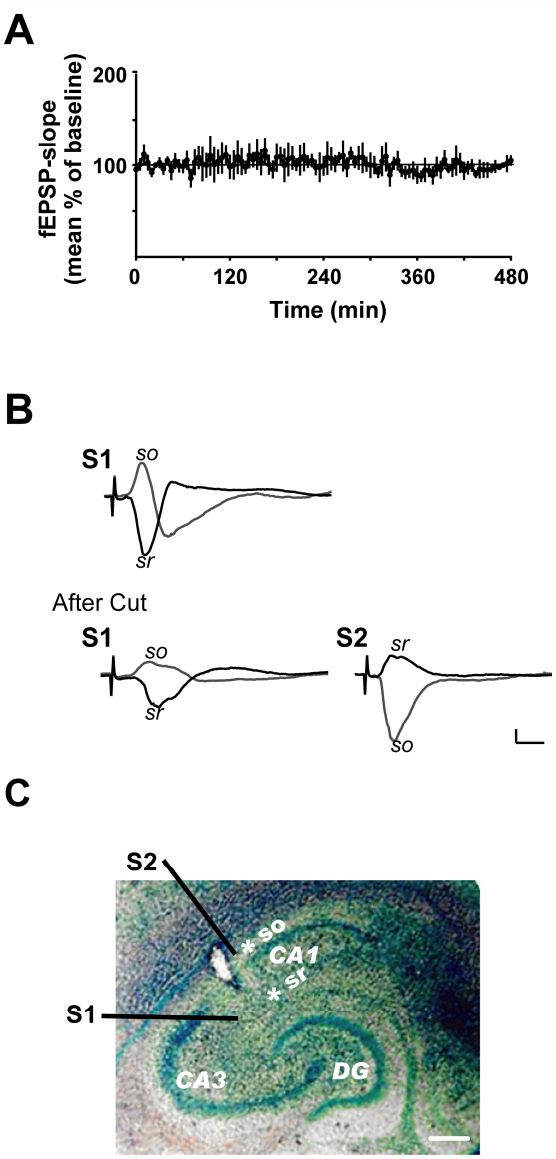

Figure 6. Baseline recordings from hippocampal-entorhinal cortex cultures. (A) The addition of cortical areas to the hippocampal formation resulted in more stable electrophysiological recordings. Field potential recordings in the CA1 area of cultured mature slices (9 DIV) showed that the fEPSP slope values remained stable during 8 h baseline recordings (at 120 min, 100.02 ± 12.20; at 480 min, 101.43 ± 4.45; n=4). (B) Electrotonic components of the stratum radiatum fEPSPs were analyzed by placing a cut through the striatum oriens fibres that project from the CA3 pyramidal cells to the CA1 basal dendrites. Analog traces of extracellularly recorded stratum radiatum fEPSPs evoked by Schaffer collateral stimulation at S1 prior to cut (*top*). Analog traces of extracellular fEPSPs recorded following a cut in the stratum oriens (*bottom)*. (C) A representative fuchsine acid stained cultured slice (14 DIV) shows the stimulation and recording sites used to obtain laminar field potentials before and after cutting the stratum oriens. The recorded sites were in stratum radiatum (sr) and stratum oriens (so; indicated by *white asterisks*) stimulation electrodes were placed either in stratum radiatum (S1) or stratum oriens (S2). The stratum oriens contribution to the stratum radiatum fEPSP is absent after the cut (*B*, *bottom left*). The stratum oriens potentials could still be evoked by stimulating proximal to the recording site. The traces illustrate that stimulation of Schaffer collateral fibers resulted in potentials generated by the activation of basal dendrite synapses in stratum oriens that electrotonically propagated to stratum radiatum where they were observed as a positive deflection in the field potentials. Scale bars: *B*, 2 mV/5 ms; *C,* 250 μm.

in stratum oriens. To determine the degree of influence that direct stratum oriens activation may have on recorded potentials in stratum radiatum, the potentials before and after a cut in stratum oriens were compared (Fig. 6b, comparing both S1 stimulations). A cut in the stratum oriens fibers resulted in a loss of the delayed positive

component at the stratum radiatum recording site, confirming that an electrotonic component in the stratum oriens contributed to the potentials recorded in stratum radiatum. One likely explanation is that the positive potentials were generated by the activation of basal dendritic synapses in stratum oriens and had electrotonically propagated to stratum radiatum where they were observed as a positive deflection in the field potentials (Fig. 6b, c). In theory the positive component of the stratum radiatum field potential could be due to the direct activation of inhibitory conductances in stratum radiatum. However, if this was the case then one would expect that the shape of the field potential in stratum radiatum would not change after cutting stratum oriens fibers. In fact, moving the stimulation electrode closer to the recording site in subsequent experiments resulted almost exclusively in field potentials with only a negative component (compare Fig. 6b, S1 before cut to subsequent experiments) further suggesting that electrotonic components from the stratum oriens had contributed when using the more distant stimulation site in stratum radiatum.

The cellular synaptic arrangement of the hippocampal circuit has been shown to be well preserved for extended periods in culture (Xiang et al., 2000). It was tested whether these synapses remain functional in mature slices cultured for 14 DIV by recording responses throughout the hippocampal circuit after stimulating in the dentate gyrus (Fig. 7a, b). Stimulation in the hilus of the dentate gyrus evoked an antidromic spike from the dentate granule cell layer. Stimulation of the molecular cell layer of the dentate gyrus evoked population spikes in the CA3 and CA1 pyramidal cell layers, which had an average amplitude of 2.7 ± 0.3 mV (n=10) and increased in latency throughout the circuit (Fig 7c, d). Similar results were obtained for slices cultured for up to 30 DIV (Fig. 7b), suggesting that the trisynaptic cascade remains functional for extended time in vitro.

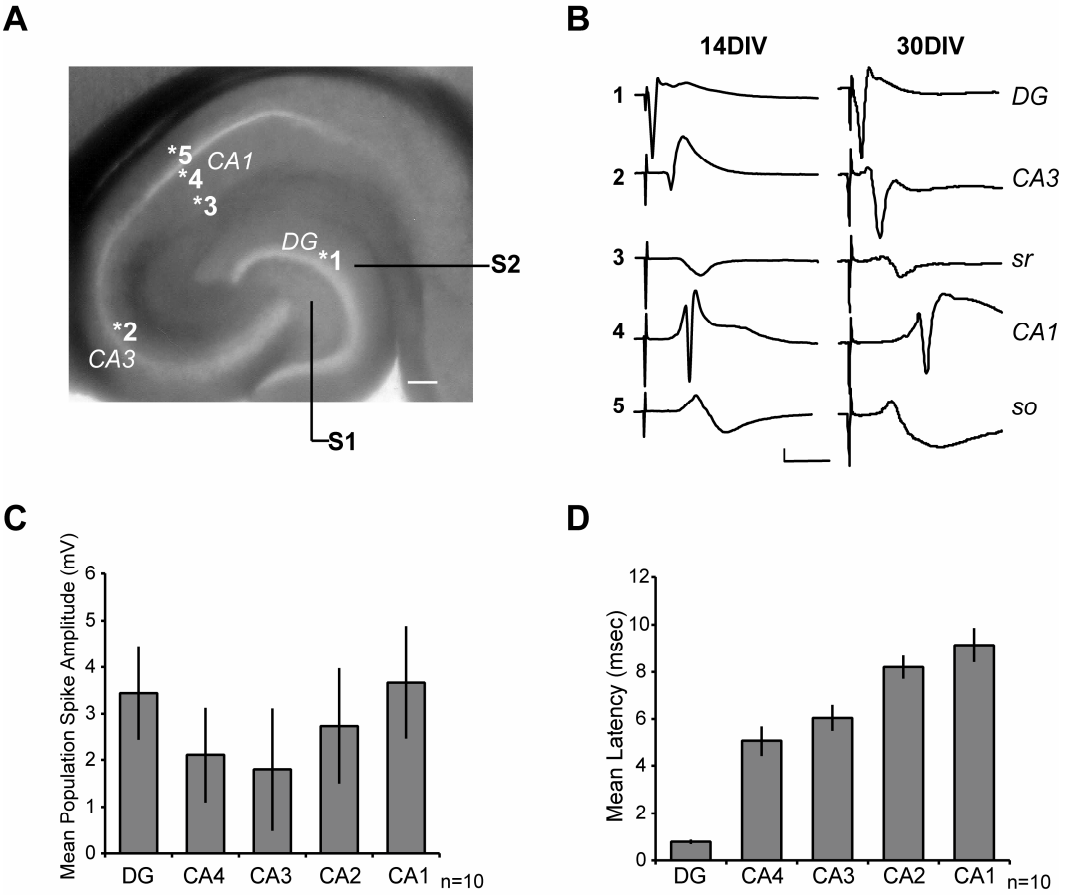

Figure 7. Mature hippocampal-entorhinal cortex slice cultures retain functional synapses throughout the hippocampal trisynaptic cascade for extended time in culture. (A) Transmitted light image of a mature hippocampal-entorhinal cortex slice cultured for 14 DIV. Neuronal layers appear as bright transparent laminae, whereas axon pathways appear dark. The image illustrates stimulation and recording sites used to obtain field potential recordings throughout the hippocampal trisynaptic cascade. (B) Analog traces of extracellular field potentials recorded in the dentate gyrus (DG), CA3 and CA1 of mature slices cultured for either 14 DIV or 30 DIV. Field potential recordings were evoked using two stimulation sites, S1 and S2, with recording sites labeled 1-5. Hilar stimulation, S1, evoked an antidromic population spike from the dentate granule cell layer (1). Stimulation of the molecular layer of the dentate gyrus, S2, evoked population spikes from pyramidal cells in area CA3 (2) and CA1 (4), as well as fEPSPs in the stratum radiatum (3) and stratum oriens (5). (C) Data represent the mean population spike amplitude recorded from slices cultured for 14 DIV, (n=10). (D) Evoked responses increased in latency throughout the circuit. Data represent the mean latency of population spikes shown in (C). Scale bars: *A*, 250 μm; *B*, 1 mV/10 ms. sr, stratum radiatum; so, stratum oriens.

It has been shown that the probability of inducing LTP at the CA1/Schaffer collateral synapse increases with the number of stimulated afferents (McNaughton et al., 1978). To evaluate the neural density in each hippocampal subarea, slices were either fixated at the time of dissection or cultured for 10-14 DIV and labeled with antibodies against the neuron-specific marker ß-III-tubulin. Immunofluorescent labeling showed that time in culture did not change the cellular organization and morphology of the hippocampal circuit. In fact, area CA1 in particular appeared to retain a large degree of its neural processes after time in culture (Fig. 8). Cell counting confirmed immunohistochemical findings and revealed that the number of CA1 neurons was reduced by 18.1 ± 6.8 % relative to acute slices (not significant). However, there was a significant reduction in the number of CA3 pyramidal neurons, which decreased to 61.1 ± 0.8 % of acute slices ($P < 0.05$). To determine whether neural loss was a result of increased serum levels in culture, mature slices were cultured in media with varying serum concentrations from 3 DIV to 14 DIV (5 %, 25 %, and 40 %). Proteins were separated using a standard SDS-PAGE gel and probed for the neuron-specific marker neurofilament 68 as an indicator of the neural density. Results revealed that the levels of neurofilament 68 protein decreased along with the increase in serum concentration (Fig. 9).

Atypical cells with irregularly shaped nuclei were observed in cultured adult slices at the border between the hilus and the inner granule cell layer (Fig. 10a, b). The identity of these cells was tested using antibodies against the neuron-specific early differentiation marker, ß-III-tubulin (Lee, 1990), the astrocyte specific marker GFAP (Debus et al., 1983), as well as the interneuron marker GAD 67. Some cells labeled only by the neuronal specific marker ß-III-tubulin were shown to project processes into the dentate molecular layer and randomly through the hilus (Fig. 10c). These cells were initially observed in 83.3 % of the slices after 8-14 DIV (n=18). However, when the protocol was altered to change the culture media to 5 % serum at 3 DIV, the occurrence of these

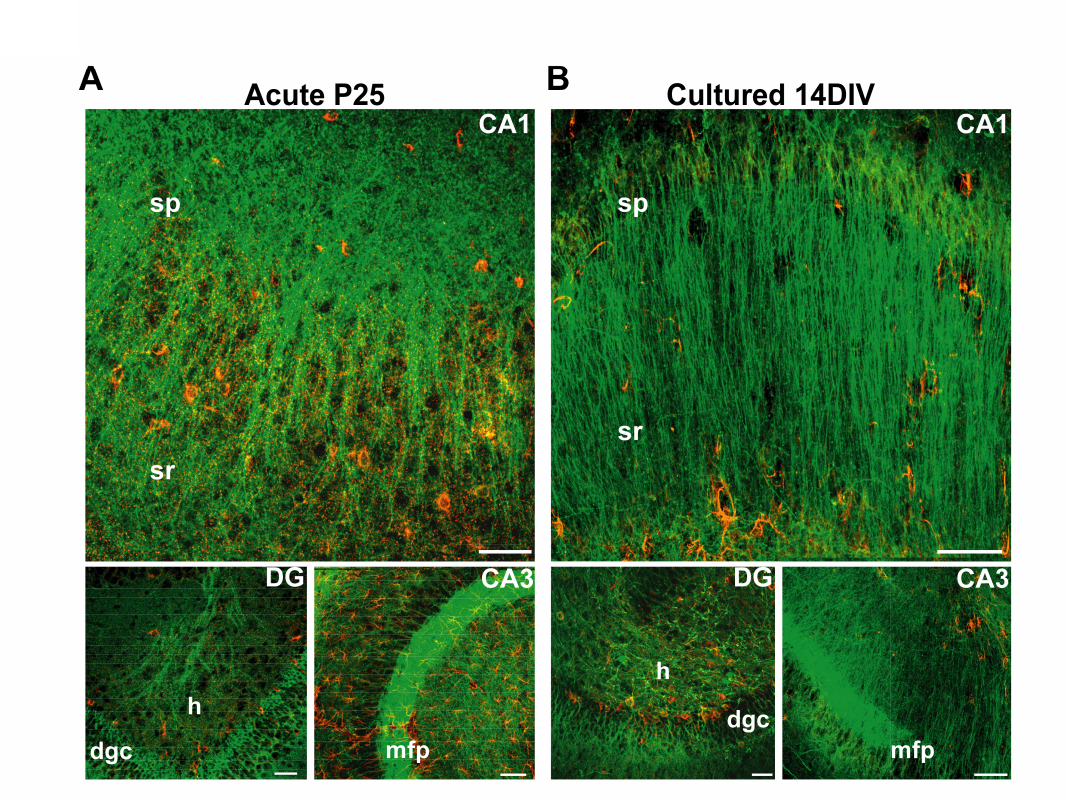

Figure 8. Pyramidal cells in the CA1 area of cultured mature hippocampal-entorhinal cortex slices retain their layered organization and neural processes. *(Top images*) In acute slices (A) and slices cultured for 14 DIV (B) cell bodies and processes of CA1 pyramidal cells *(green*) and interneurons (*red*) were visualized using immunofluorescent staining with antibodies to β-III-tubulin and glutamic acid decarboxylase (GAD 67). Note that the pyramidal cell processes in cultured slices become organized within a single optical plane as a result of culture conditions, in contrast to acute slices where processes are not limited in their spatial orientation. Neuronal cell bodies and processes in the dentate gyrus and area CA3 were also visualized (*bottom*). *A* and *B* are composites of stacked optical sections obtained by confocal microscopy. Scale bar (shown in *B*): 40 μm. *dg*c, dentate granule cell layer; *h*, hilus; *m*, molecular layer; *mfp*, mossy fiber pathway; sr, stratum radiatum; *sp*, stratum pyramidale.

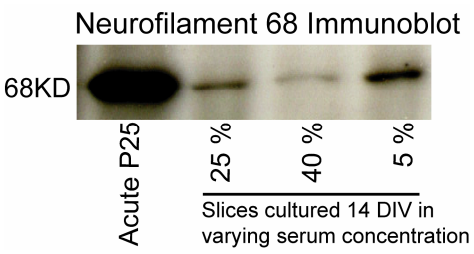

Figure 9. The neural density of mature cultured slices increases when serum concentrations are reduced. Mature slices were incubated in media with varying serum concentrations from 3 DIV to 14 DIV (5 %, 25 %, and 40 %). Immunoblot analysis of neurofilament 68, a neuron-specific marker, was used as an indicator of the neural density in acute P25 slices and P25 slices cultured for 14 DIV (n=4 for each group). Immunostaining of neurofilament 68 protein revealed the decrease of neural density in cultured compared to acute slices, as well as the lowest neural density with the highest serum concentration.

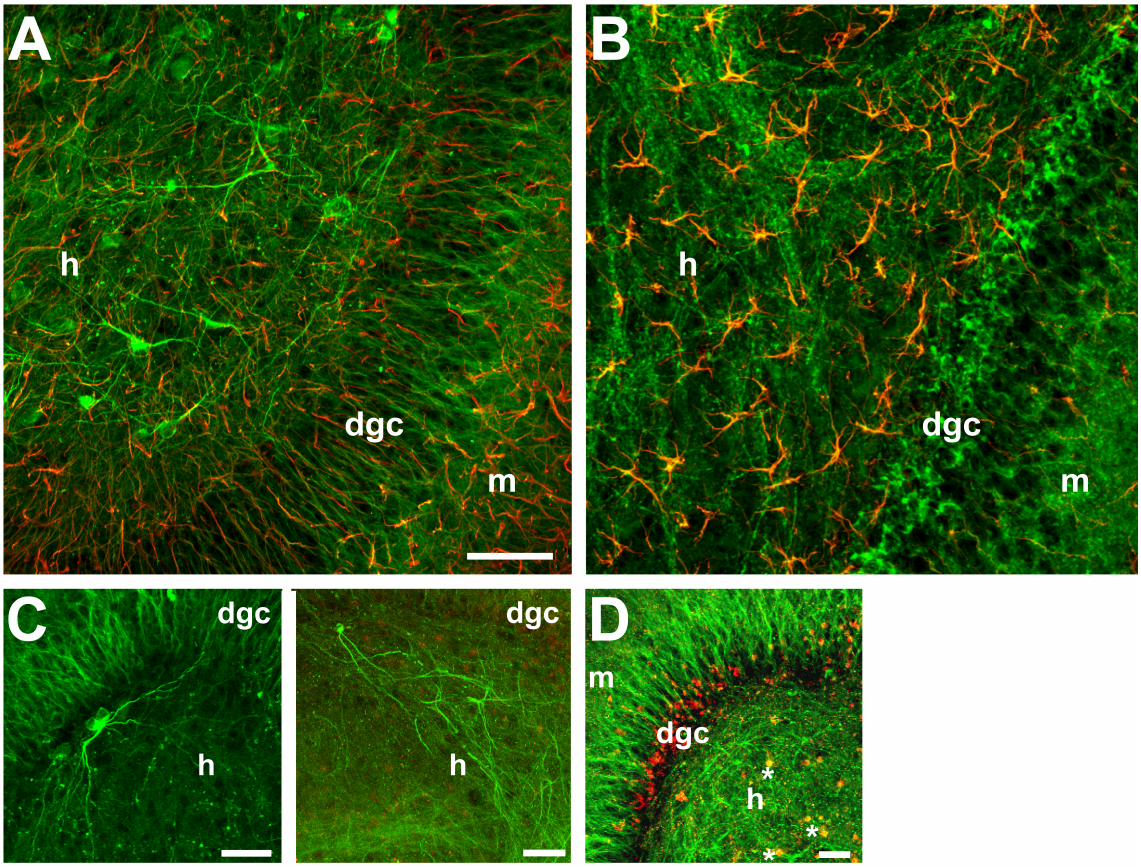

Figure 10. Ectopic ß-III-tubulin-immunoreactive cells were found in the hilus of mature hippocampal-entorhinal cortex slices cultured in 25 % horse serum. Beta-III-tubulin immunohistochemistry (*green*) illustrates a population of cells in the hilar region of mature cultured slices (A), which was not seen in acute slices from the same animal (B). For comparison glial cells were labeled using antibodies to glial fibrillary acidic protein (GFAP, *red*). (C) Displaced ß-III-tubulin-immunoreactive neurons were also shown to project erratic processes (*right and left panels*), suggesting there is some reorganization in the dentate gyrus of mature slices cultured in high serum concentrations. (D) *Right panel.* Nuclear BrdU (*red*) was co-localized with ß-III-tubulin immunostaining (*green*) in the hilar region of mature slices cultured in the presence of BrdU for 14 DIV (*white asterisks* indicate co-localization), indicating that some ß-III-tubulin-immunoreactive neurons, in the hilus of mature slices cultured in high serum, resulted from neurogenesis in culture. Note that most of the BrdU positive cells in the subproliferative zone of the denate granule cell layer are ß-III-tubulin-negative. Slices cultured in high serum were not used in further electrophysiological experiments. *A–C* are composites of stacked optical sections obtained by confocal microscopy. Scale bars: 40 μm. dgc, dentate granule cell layer; h, hilus; m, molecular layer.

cells decreased to 18.2 % of the slices (8-14 DIV, n=11) implicating high serum concentration to contribute to the presence of these neurons. Co-localization of BrdU, which is taken up by mitotically active cells, with the neuron–specific marker ß-III-tubulin showed that 36.3 % of the displaced neuronal cells were generated in culture (Fig. 10d). However, the majority of positively labeled BrdU cells were co-localized with the astrocyte-specific antibody, GFAP (data not shown).

Changes in the morphology, distribution, and number of astrocytes in mature cultured hippocampal-entorhinal cortex slices were examined using immunohistochemistry. Astrocytes cultured from animals older than post-natal day 13 have been characterized to undergo morphological and chemical changes that alter their ability to support neurons in vitro (Smith et al., 1990; Ascher et al., 1998; Howard, 2000). Such a tissue culture effect could influence the viability of mature hippocampal cultures. Slices, 10-14 DIV, were labeled with antibodies against GFAP, an astrocyte-specific cell marker. Staining revealed that astrocytes within cultured mature slices alter their morphology, lose their random and even distribution throughout the hippocampal lamina, and increase in number (Fig. 11a, b). Cell counting experiments revealed that astrocytes in area CA1 of the hippocampal slice increased by 53.1 ± 2.5 % after 14 DIV. In addition, astrocytes from a small portion of mature hippocampal cultures became reactive as indicated by a characteristic change in morphology as well as the expression of chondroitin sulfate proteoglycans (CSPGs, Fig. 11c, d) known to change the extracellular cell matrix (McKeon et al., 1991; Hoke and Silver, 1994; Silver, 1994), resulting in excitotoxicity (Barger and Basile, 2001), a loss of neuronal support (Smith et al., 1990; Howard, 2000), as well as the production of inflammatory cytokines (Silver, 1994; Chao et al., 1995; Viviani et al., 1998), all of which could influence LTP.

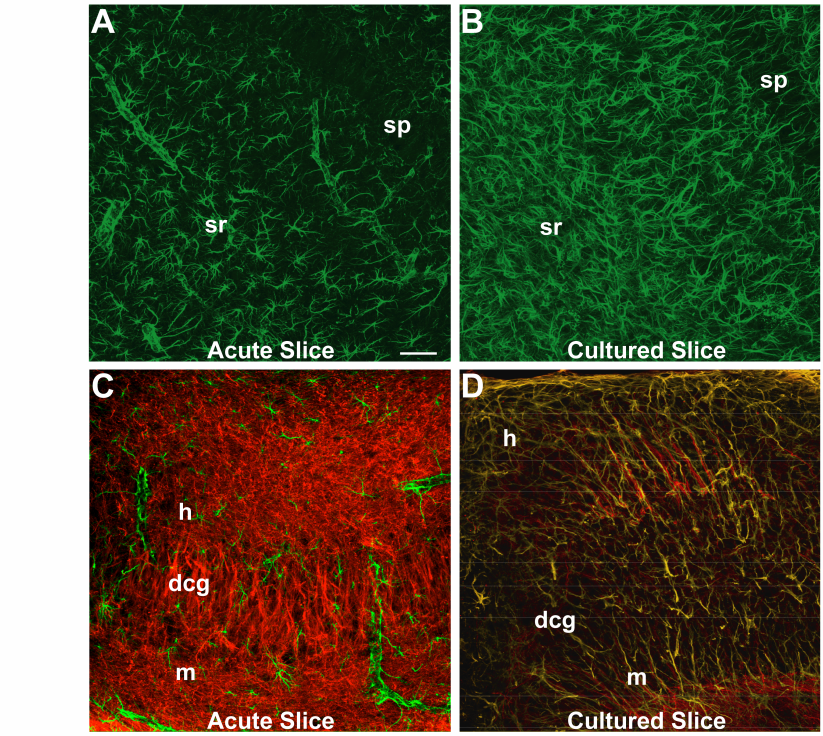

Figure 11. Astrocytes in cultured mature slices alter their morphology, lose their even distribution throughout the hippocampal lamina, and increased in number as indicated by a comparison of *A* and *B*. Immunofluorescence using antibodies to glial fibrillary acidic protein (GFAP), an astrocyte specific cell marker, labeled the entire cell body of astrocytes in area CA1 of acute slices (A) and slices cultured for 14 DIV (B). (C) In the dentate gyrus of acute hippocampal slices GFAP (*green*) was not co-localized with chondroitin sulfate proteoglycans (CSPGs, *red*) illustrating that astrocytes do not express CSPGs. Note: CSPGs are normally expressed by neuronal axons in the dentate and mossy fiber pathways in vivo (Engel et al., 1996) as shown in the CSPG expression pattern after immunostaining in the acute slice. (D) CSPGs (*red*) were co-localized with GFAP immunostaining (*green*) in the dentate gyrus of select slices cultured for 14 DIV (*yellow* indicates co-localization) suggesting that astrocytes have become reactive. *A-D* are composites of stacked optical sections obtained by confocal microscopy. Scale bar: 40 μm. *sr*, stratum radiatum; sp, stratum pyramidale; *dg*c, dentate granule cell layer; h, hilus; m, molecular layer.

3.1.2. Characterization of the fEPSP in the CA1 Stratum Radiatum

Increases in synaptic strength, which are thought to participate in information processing at the cellular level, can be measured as an increased fEPSP slope. To

determine whether persistent synaptic potentiation can be induced and maintained in cultured mature hippocampal-entorhinal slices, the fEPSP recorded at the Schaffer collateral input to the pyramidal neurons of the CA1 region was characterized. Synapses were tested using a paired-pulse paradigm that resulted in paired-pulse facilitation, i.e. an increase in the slope of the second response during paired stimulation. The paired-pulse facilitation, which is short lasting and presumably presynaptic, indicates that there is sufficient transmitter release from the presynaptic afferents even when pulses are given at short intervals. On the other hand, when CA1 pyramidal cell population spikes were recorded simultaneously, the second response decreased for paired stimuli (Fig. 12a, b). This pattern suggests that culture conditions do not lead to synaptic failure by, for example, the depletion of readily releasable vesicles.

It was further investigated whether NMDA currents contribute to the fEPSP to show that the receptors and their excitatory synaptic currents, which are essential for the induction and expression of LTP are not lost after time in culture. Receptor antagonists were added in succession until the complete fEPSP was blocked to reveal the contribution of each receptor type to the total fEPSP. First, the addition of picrotoxin (30 μM), a $GABA_A$ channel antagonist, resulted in temporary synchronized bursting of CA1 and CA3 pyramidal cells indicating the presence and functionality of the inhibitory synaptic circuit. The remaining excitatory synaptic current was expected to be composed of an earlier AMPA component, and a smaller late NMDA component (Watkins and Evan, 1981; Hestrin et al., 1990). Accordingly, the AMPA/kainate receptors antagonist NBQX (10 μM), resulted in a large reduction of the evoked fEPSP amplitude, and the NMDA receptor antagonist CPP (10 μM) completely blocked the remaining signal (Fig. 12c, n=4 slices). The amplitude component that remained as a result of NMDA receptor activation corresponded to 33.88 ± 12.39 % of the combined AMPA and NMDA-dependent fEPSP amplitude.

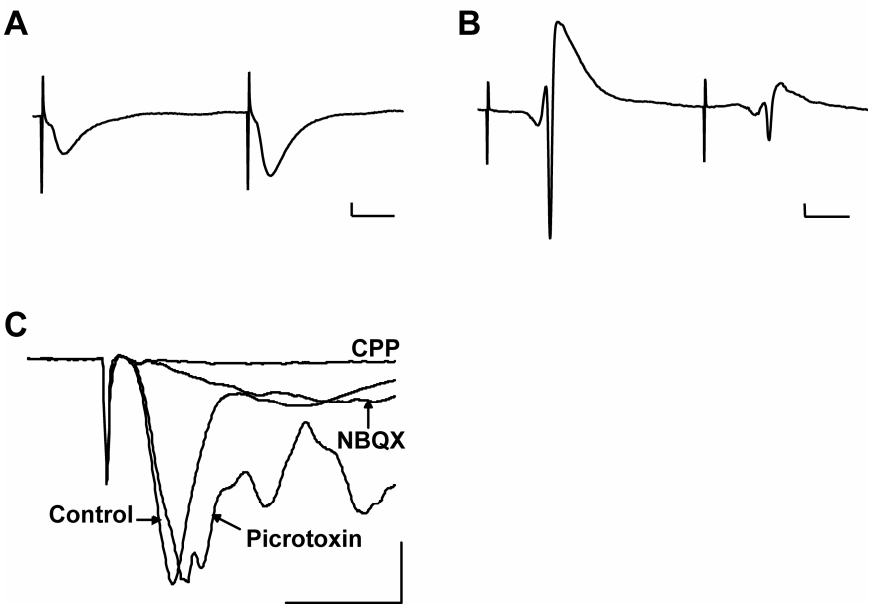

Figure 12. The synapses of CA1 pyramidal cells in mature slice cultures show functional properties corresponding to acute transverse slices. (A) and (B) Analog traces of extracellularly recorded field potentials when using paired-pulse stimulation in stratum radiatum. (A) Stimulation resulted in paired-pulse facilitation of the fEPSP evoked at the synapses between the Schaffer collateral axons and apical dendrites of CA1 pyramidal cells, and (B) simultaneously in a paired-pulse depression of the CA1 pyramidal cell population spike amplitude. (C) NMDA receptor activity at the apical dendrites of CA1 pyramidal cells can be measured physiologically. Representative analog traces showing the stratum radiatum fEPSP after adding the following antagonists. After the addition of picrotoxin (30 μM), the AMPA receptor contribution was blocked using NBQX (10 μM), and the remaining NMDA component was completely eliminated using CPP. Arrows match each additional antagonist application to the response recorded thereafter. Scale bars: *A* and *B*, 0.5 mV/10 ms; *C*, 0.5 mV/5 ms.

3.1.3. Induction and Expression of LTP in Area CA1 of Cultured Mature Hippocampal-Entorhinal Cortex Slices.

The stimulation requirements for LTP induction and its maintenance for more than 4 h at the Schaffer collateral input to the CA1 pyramidal neurons was investigated in cultured mature hippocampal-entorhinal cortex slices. I tested three different high-frequency stimulation paradigms to determine whether long-term modifications of synaptic inputs were possible in mature cultured hippocampal-entorhinal cortex slices.

The results were categorized into instances with potentiated (> 20% increase at 1 min posttetanus compared to baseline) and non-potentiated fEPSPs, and potentiated slices were further classified by the LTP duration (Table 1). Three or more trains of high-frequency stimulation consistently resulted in after-burst discharges and synaptic depression following the third stimulus train. When two rather than three stimulus trains were given, bursting was reduced to 38.8 % of the slices (n=18). LTP induction was significantly more successful using the 100 Hz or 200 Hz protocols compared to using theta burst stimulation (100 Hz, 61.1 % of the slices, n=18; 200 Hz, 55 %, n=20; theta burst stimulation, 27 %, n=15). However, 100 Hz stimulation less often resulted in after-burst discharges and subsequent depression. The 100 Hz protocol consisting of 2 trains of 100 pulses at 100 Hz with a 10 min intertrain interval was therefore adapted for further LTP experiments.

Table 1. LTP trials in mature slice cultures [a]

Stimulation Protocol	*PTP*	*E-LTP < 4 h*	*L-LTP > 4 h*	*ABD*	*NR*	n
Theta Burst	0	3	1	7	4	15
200 Hz	4	5	2	7	2	20
100 Hz:	4	7	0	5	2	18
5 % serum	6	11	0	0	3	20
Enhanced chamber	2	3	4	0	1	10
Single animal		1	5			6

[a] Slice cultures were maintained in vitro for 10-15 days, after which one of the listed stimulus paradigms was tested for the success of LTP induction and expression. Increases of greater than 20 % compared to baseline (at 1 min post tetanus) were considered as potentiation and those cases were further divided by duration. Posttetanic potentiation (PTP) was defined as a return to baseline after less than 5 min, E-LTP lasted more than 30 min but less than 4 h and L-LTP was defined as potentiation that lasted more than 4 h. Tetanization that did not result in potentiation was separated into non-responsive (NR) cases or cases with after-burst discharges (ABD), which were often accompanied by depression. Experiments with the 100 Hz stimulation protocol are subdivided to reflect further changes in culturing and recording protocols, which include a decrease in serum concentration at 3 DIV, an enhancement of the recording chamber, and a comparison of slices taken from a single animal to control for a possible lesser variation within a dissection.

To reduce the number of LTP trials that resulted in spontaneous bursting and depression and to increase the duration of LTP expression, it was tested whether serum may be excitotoxic to hippocampal neurons in culture at high concentrations (see Ye and Sontheimer, 1998), which could contribute to the frequent failure of LTP induction and/or maintenance in cultured mature slices. Reducing the serum concentration from 25 to 5 % at day 3 in vitro resulted in an increase in the frequency of slices with successful LTP induction to 85 %, and in addition, the absence of bursting (Table 1). These results suggest that the higher serum concentrations after 3 DIV had previously increased the likelihood for the failure of LTP induction.

The 100 Hz stimulation protocol in mature cultured slices had consistently resulted in E-LTP lasting less than 4 h. To rule out the contribution of unfavorable recording conditions to short-lasting LTP, the recording chamber was modified to better support cultured slices by introducing enhanced humidification with a vapor saturation of $\geq$ 90 %. Slices remained viable in the improved recording chamber for extended periods of time as shown by stable fEPSP baseline recordings for a 14 h period after which LTP was successfully induced (data not shown). In addition, the overall frequency of an extended LTP duration was increased with the improved chamber conditions, in which long-lasting LTP occurred in 40 % of all slices (n=10, see Table 1). Although long-lasting, the induced LTP consistently had a decremental component, with a declining potentiation for 3 h and a subsequent plateau at an ~ 30 % increase compared to baseline. Potentiated responses were observed to remain significantly increased for up to 6 h (the longest time point measured; mean at 360 min, 132.89 ± 13.79 %, $P < 0.05$, n=4). Along with long-lasting LTP in 40 % of the slices, we observed E-LTP in 30 % of all slices when using the same stimulus protocol and recording conditions (mean at 60 min, 130.67 ± 8.71 %, $P < 0.05$; mean at 120 min, 115.64 ± 12.18 %; n=3; Table 1 and Fig. 13a). All slices that showed E-LTP rather than L-LTP were characterized by smaller fEPSP amplitudes during

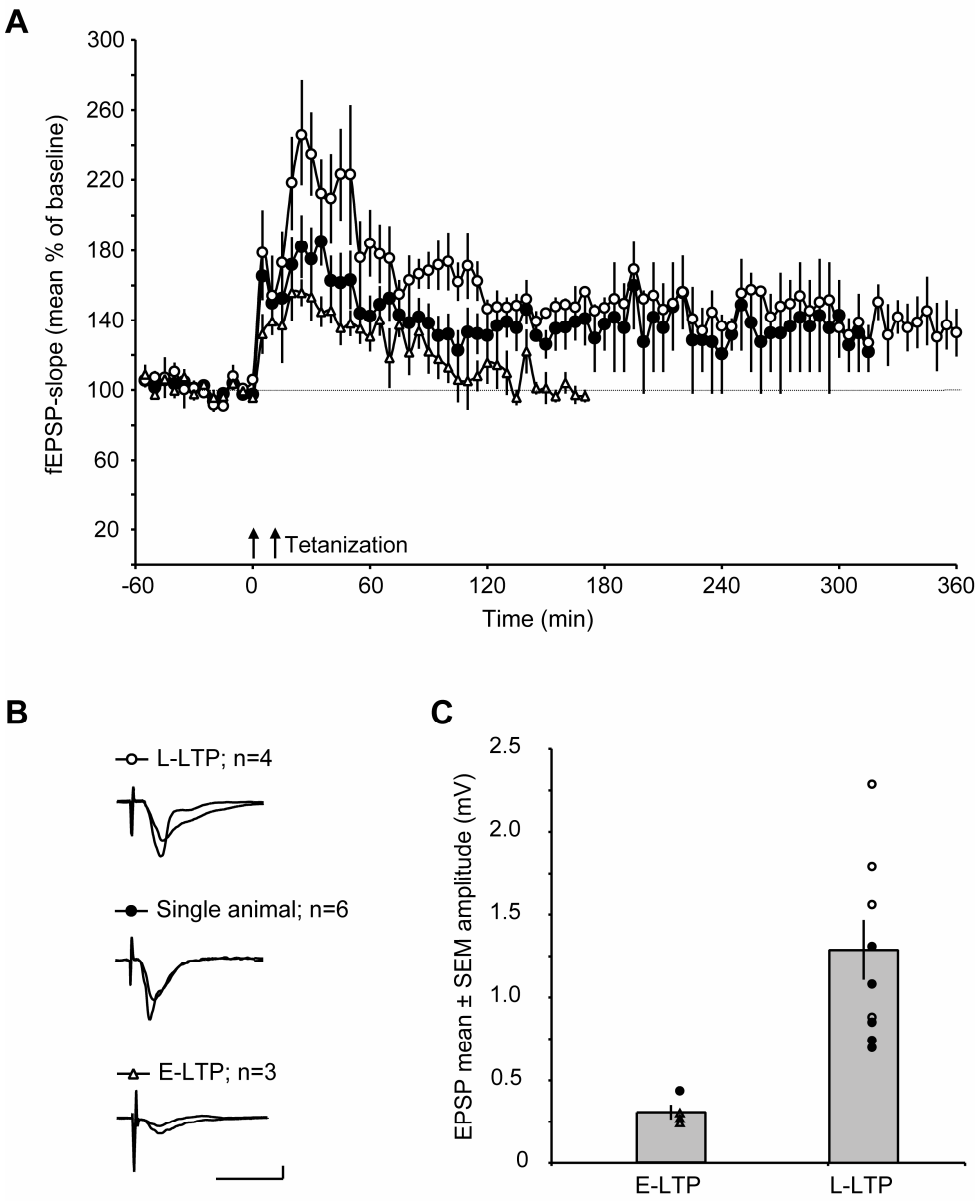

Figure 13. Expression of LTP in the CA1 synapses of cultured mature hippocampal-entorhinal cortex slices following high-frequency stimulation (HFS; 2 trains of 100 Hz for 1 sec, 10 min intertrain interval). HFS of slices with smaller fEPSP amplitudes resulted in E-LTP, whereas the same stimulation paradigm resulted in L-LTP in slices with larger fEPSPs. (A) HFS (arrows) of the Schaffer collaterals resulted in both L-LTP (o), and also E-LTP (Δ) in mature slices (L-LTP at 360 min, 132.89 ± 13.79 %, n=4), (E-LTP at 60 min, 130.67 ± 8.71 %; at 120 min, 115.64 ± 12.18 %; n=3). HFS induced L-LTP in 5 of 6 cultured slices from the same animal (•; at 300 min, 142.8 ± 20.37 %, n=6). Symbols in *A* also correspond to the same data represented in *B* and *C*. (B) Superimposed representative field potentials taken 10 min before and 30 min after HFS of Schaffer collateral axons. Note that tetanization experiments that resulted in E-LTP were characterized by smaller fEPSP amplitudes during baseline recordings. (C) Mean baseline fEPSP amplitudes for each trial shown in (A) that resulted in E-LTP (< 4 h) and L-LTP (> 4 h). Symbols represent the mean baseline fEPSP. Scale bar: 0.5 mV/20 ms.

baseline recordings (Fig. 13b, c), suggesting that the failure of LTP to last longer than 4 h was related to the lower initial amplitudes of the fEPSP.

To investigate whether the variation in the response to 100 Hz stimulation was due in part to differences between dissections, LTP was induced in slices taken from the same animal (right and left hemispheres, n=6, see Table 1). High-frequency stimulation resulted in an average initial potentiation of excitatory postsynaptic potentials at ~ 150 % of the baseline slope and with a minimum duration of at least 3 h when using the 100 Hz stimulus protocol (mean percent of baseline at 60 min, 142.81 ± 11.94 %, $P < 0.05$; at 300 min, 142.81 ± 20.37 %, $P < 0.05$, n=6, Fig. 13a). As in previous experiments a robust short-term LTP was observed along with a weaker and more variable longer-lasting LTP. The variation in response to strong stimulation was reduced between slices from the same animal with all cultured slices showing a similar initial magnitude of potentiation, and longer-lasting LTP in 83 % of all slices.

3.1.4. LTP in area CA1 is NMDA Receptor-Dependent

I have shown that mature hippocampal-entorhinal cortex cultures retain synaptic properties in vitro that allow for the induction and expression of long-lasting LTP. LTP induced by 100 Hz stimulation requires postsynaptic Ca^{2+} influx and is either dependent on NMDA receptor activation (Collingridge et al., 1983; Lynch et al., 1983) or on the activation of voltage-gated Ca^{2+} channels (Grover and Teyler, 1990, 1995). It was tested whether LTP induced by 100 Hz stimulation of mature hippocampal slices is NMDA receptor-dependent. When 100 Hz stimulation was applied to the Schaffer collateral fibers in the presence of the NMDA receptor antagonist APV (50 μM), the potentiation was significantly reduced relative to control LTP ($P < 0.05$, Fig. 14). In the presence of APV, the stimulation resulted in an immediate posttetanic potentiation (mean percent of

baseline at 10 min (126.31 ± 10.81 %, n=6) but was significantly different from controls at 60 min ($P < 0.05$, mean APV percent of baseline, 105.69 ± 6.07 %, n=6; control, 142.04 ± 10.68 %, n=6) indicating that the long-lasting LTP induced by 100 Hz stimulation in mature hippocampal-entorhinal cultured slices was NMDA receptor-dependent.

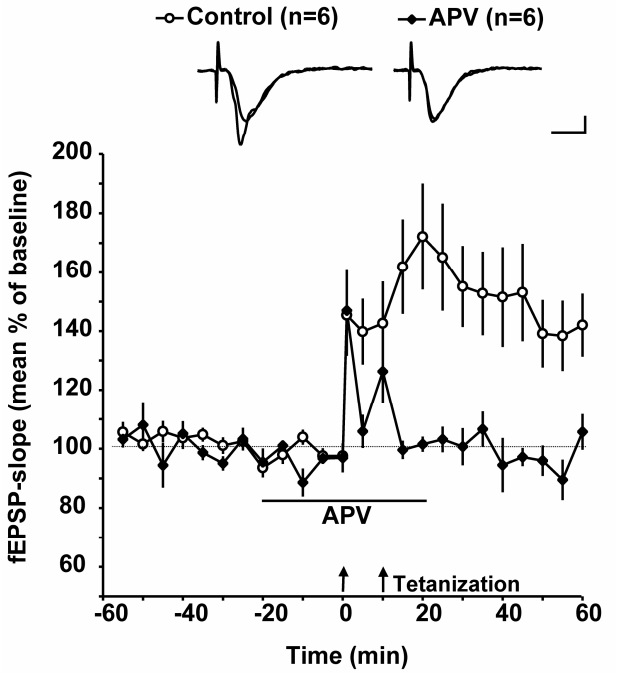

Figure 14. LTP in the CA1 area of mature cultures is NMDA receptor dependent. APV was added to ACFS and superfused for 20 min before until 20 min after the induction of LTP (*bar*). High-frequency stimulation (HFS; *arrows*) applied in the presence of 50 μM APV (♦), resulted in significantly lower initial potentiation than HFS applied in control conditions (O; APV at 60 min, 105.69 ± 6.07 %, n=6, Control at 60 min, 142.04 ± 10.68 %, n=6). *Top*: Superimposed representative field potentials in the CA1 area at 10 min before and 30 min after HFS of the Schaffer collaterals in normal ACSF (control; data also included in Fig. 13), or in ACSF with 50 μM APV. Scale bar: 0.5 mV/10 ms.

3.2. Discussion of Mature Hippocampal Slice Characterization

3.2.1. Improved Electrophysiological Signals and their Stability in Mature Hippocampal Slice Cultures

By improving the culturing procedures of hippocampal-entorhinal slice cultures from mature animals (P25-30), it was possible to obtain slices with a functionally intact trisynaptic loop, stable fEPSP amplitudes during baseline recordings, and reliable LTP

including a long-lasting form of LTP. LTP in mature hippocampal-entorhinal cortex slices after 100 Hz stimulation of the Schaffer collateral input to the CA1 pyramidal neurons was NMDA receptor-dependent, characterized by an initial decremental component, and could last for up to 6 h (the longest time point measured). LTP induction and maintenance in mature cultured slices was dependent on optimizing the culture conditions to obtain adult tissue cultures with electrophysiological properties that resemble those of acute slices and, in part, intact animals including the capability for LTP induction by extracellular stimulation.

The use of mature hippocampal-entorhinal cortex slice cultures in electrophysiological experiments required five key protocol changes. First, it was found that using a vibratome to prepare hippocampal-entorhinal slices for culture resulted in a higher proportion of slices with undamaged CA3 or CA1 regions. The use of a modified vibratome allows one to constantly keep the tissue at 4 °C throughout the entire procedure and to take sections along the entire hemisphere from which the hippocampus and associated subicular and entorhinal cortices can be removed by a single cut, which minimizes tissue handling and potential cell damage. Second, adding the adjacent subicular and entorhinal cortices to the hippocampus greatly enhanced the stability of baseline recordings by reducing the occurrence of spontaneous activity within the hippocampal circuit, which permitted stable baseline recordings (Fig. 6a). Third, slices incubated overnight at 34 °C in carbogen retained larger electrophysiological responses and recovered better from initially low synaptic amplitudes (at 10 DIV) in comparison to slices maintained at ambient O_2 (Fig. 5). Fourth, lowering the serum concentrations had a dramatic effect on the capability for LTP induction in mature hippocampal-entorhinal cortex co-cultures and was necessary to reliably induce LTP in mature cultured slices. Reducing the serum concentration from 25 to 5 % at 3 DIV resulted in a significant increase in the frequency of LTP induction to occur in up to 85 %

of all slices in the absence of bursting and synaptic depression following 100 Hz stimulation (see Table 1). Finally, modification of the recording chamber resulted in an overall increase in the duration of LTP (> 4 h) with long-lasting LTP occuring in 40 % of all tested slices (Table 1). Taken together, improved electrophysiological characteristics and the success of LTP induction and maintenance were shown to depend on adapted methodological procedures aimed at better sustaining the survival of hippocampal neurons and their connections, which is a prerequisite for sustained synaptic function and the induction of synaptic plasticity in mature cultured slices.

3.2.2. LTP in Mature Hippocampal-Entorhinal Cortex Slice Cultures

After 2-3 weeks in culture, mature (P25-30) rat hippocampal-entorhinal cortex slices are capable of activity-dependent changes in synaptic strength at the Schaffer collateral input to the pyramidal neurons of the CA1 region. Further investigation of the properties of LTP in culture after high-frequency stimulation showed that high-frequency stimulation (100 Hz) at the Schaffer collateral input to the CA1 pyramidal neurons in mature hippocampal-entorhinal cortex slices is NMDA receptor-dependent and consistently characterized by an initial robust early phase of LTP that declines and is followed by a variable late phase. In acute slices a single high-frequency stimulation train (100 Hz) consistently evokes E-LTP. The early-phase of LTP is short-lasting, stable for up to 3-4 h, dependent on NMDA receptor activation, and maintained by second messenger cascades activated after an initial influx of Ca^{2+} (reviewed in Malenka and Nicoll, 1999; Soderling and Derkach, 2000). When three or more high-frequency stimulus trains are given a second component is added, which begins gradually during the first 1-3 h and is retained as long as 10 h in acute slices (Frey et al., 1988, 1993, 1996; Matthies et al., 1990). The late phase of LTP is protein synthesis dependent (Krug et al., 1984; Frey et al., 1988; Huang and Kandel, 1994; Nguyen et al., 1994; Huang et al., 1996; Frey et al.,

1996), activated by second messenger cascades typically initiated during E-LTP, and prevented in area CA1 in the presence of dopamine antagonists during its induction (Frey et al., 1990; Frey et al., 1991). Co-activation of subcortical inputs, such as dopamine, is necessary to establish the late forms of LTP in area CA1 (Frey et al., 1990; Frey et al., 1991; Huang and Kandel, 1995; Swanson-Park et al., 1999).

High-frequency stimulation (100 Hz) of the Schaffer collateral/ CA1 synapses in mature hippocampal-entorhinal slices resulted in varied amplitudes and time courses of LTP, even after introducing the modified protocol that improved and prolonged LTP (Table 1 and Fig. 13a). The stimulus protocol that had been identified to be the most effective (2 trains of 100 pulses at 100 Hz, 10 min intertrain interval) resulted in at least an initial potentiation in 70 % of all slices. The potentiation was characterized by an initial robust E-LTP and, in 40 % of the slices, also a form of L-LTP. An analysis of the fEPSP amplitudes during baseline recordings revealed that all slices that only expressed E-LTP had lower fEPSP amplitudes than any of the slices that showed L-LTP (Fig. 13b, c). These results suggest that the expression of L-LTP was prevented when fewer synapses and/or lesser currents at each synapse contributed to the fEPSP indicating that the size of the fEPSP can predict whether L-LTP can be induced in a slice culture. Even when expressed, the long-lasting LTP in mature cultured slices was weak in comparison to the early phases and of varying duration between slices (Fig. 13a). The long-lasting LTP observed in mature cultures slices does not reflect the same non-decremental transition from E-LTP to L-LTP shown in acute slices. Cultured slices lack afferent innervation from extrinsic inputs, which may be required to establish the late phases of LTP at maximal levels of potentiation. Further investigation is needed to determine factors that modulate the late form of LTP in mature hippocampal slices. Thus, it remains currently open whether the prolonged form of LTP in such cultures also depends on protein synthesis.

Mature hippocampal-entorhinal cortex slice cultures were shown to be hyperexcitable, in particular in response to the high-frequency stimulation needed to induce long-lasting changes in synaptic strength. Three or more trains of high-frequency stimulation have been shown to consistently induce L-LTP in acute slices (Frey et al., 1993; Nguyen et al., 1994). The same stimulus pattern resulted in after-burst discharges and synaptic depression in cultured mature slices. With a reduction to 2 stimulus trains it was possible to induce LTP within a portion of cultured slices (see Table 1).

It was shown that the surviving hippocampal circuit, that has been established in P25 animals in vivo, remains functional in mature cultured slices for at least up to 30 DIV (see Fig. 7). In addition, I show that a distinct balance between excitation and inhibition is maintained, and the proper receptor contribution to the fEPSP is retained when the slices are cultured in low serum media (Fig. 12c). However, strong stimuli resulted in spontaneous bursting and oscillations throughout the hippocampal circuit. Such hyperexcitability may in part be due to intact functional connections throughout the hippocampal trisynaptic cascade, or differences in inhibition. In acute slices, severed afferent fibers are stimulated, preventing high-frequency stimulation from propagating for long distances. In mature cultures only intact, healthy neurons and their connections are retained and fiber stumps can be assumed to have degenerated. LTP induction in mature slices is thus complicated by the delicate balance between providing a high enough extracellular stimulus intensity to activate a sufficient number of afferent axons to the CA1 cell population without hyperexciting the entire circuit. Similar problems for LTP induction have also been described at the CA1 synapses in the intact animal (Leung, 1979; Leung and Shen, 1995). Therefore, in contrast to acutely prepared adult hippocampal slices, the properties of LTP induction in mature cultured slices show properties that are otherwise only observed in vivo with a completely connected hippocampal-entorhinal cortex loop.

The Role of Serum in the Induction of LTP. Impaired LTP induction in cultured mature slices occurred as a direct consequence of cell culture conditions, most importantly the serum content in the culture media. When the serum concentration was reduced from 25 to 5 % at 3 DIV, bursting and depression following 100 Hz high-frequency stimulation was eliminated and the occurrence of long-term potentiation significantly increased to occur in 85 % of all slices (see Table 1). Possible mechanisms by which serum prevents LTP induction are by decreasing the total neural density of the mature slice (see Fig. 9) and by inducing the occurrence of ectopic neurons (see Fig. 10).

It was shown that the neural density of mature hippocampal-entorhinal cortex slices cultured for 14 DIV decreased with increasing serum concentration in the culture media (see Fig. 9) suggesting a direct effect of serum on the survival of hippocampal neurons or their processes in culture. Decreased serum concentration, along with increased neural density resulted in successful LTP induction in most slice cultures. The probability of inducing LTP at the Schaffer collateral/CA1 synapse increases with higher stimulation intensities that recruit a larger number of axons (McNaughton et al., 1978). Cell counting experiments revealed that neuronal loss, relative to acute slices, after 14 DIV in 25 % serum was most pronounced in the CA3 area. A significant reduction in CA3 pyramidal cells can in turn lead to a reduction in the number of axons projecting to CA1 pyramidal cells, thus reducing the probability to induce LTP at the Schaffer collateral/CA1 synapses. A reduced density and number of surviving neurons in culture along with a decreased number of afferent fibers recruited by high-frequency stimulation can result in the failure to provide the spatiotemporal cooperativity for LTP induction at the Schaffer collateral-CA1 pyramidal cell synapse (McNaughton et al., 1978; Kelso and Brown, 1986; Brown et al., 1990; Bliss and Collingridge, 1993; Bliss et al., 2003). Such properties of LTP induction could also explain the variability in the resulting magnitude of

LTP between slices as well as the difference in the time-course of LTP expression (see Fig. 13 and Table 1). Most fibers in the vicinity of the stimulating electrode can be assumed to be activated by stimulation in slice cultures, which are only 100-200 μm thick after 10 DIV leading one to believe that the variability in fEPSP amplitude reflects variability in the number of activated afferent fibers (see Fig. 13b, c). Small differences in the dissection procedure during each slice preparation are expected to lead to varying levels of neural density between slice preparations and, to a lesser extent, between slices from one animal.

Increased serum concentrations were also shown to generate ectopically placed neurons within the hilus of cultured mature hippocampal-entorhinal cortex slices in vitro (see Fig. 10). The reduction in serum concentration reduced the occurrence of ectopic neurons from 83.3 to 18.2 %. The appearance of such cells in vitro could be a result of epileptiform activity (oscillations, bursting) induced, for example, by serum-borne glutamate or by spreading of the neuronal cell layers in culture. In adult rats ectopic granule cells located at the border of the hilus and the inner granule cell layer are characterized by irregular shaped nuclei (Parent et al., 1996, 1999) and give rise to abnormal axonal projections resulting in hippocampal hyperexcitability in vivo (Scharfman et al., 2000). These ectopic granule cells are generated as a result of kainic acid-induced seizures and resemble those in mature cultures both in morphology and immunofluorescent labeling (Fig. 10 a, d). Although we did not test whether these cells form connections influencing the properties of CA3 pyramidal cells as reported in vivo (Scharfman et al., 2000), we observed that spontaneous bursting and after-burst discharges were reduced along with their reduced occurrence. Moreover, fluorescent imaging revealed some displaced granule cells that projected misguided processes (see Fig. 10c). Xiang et al. (2000) also mentioned the appearance of dislocated cells from the granule cell layer, and confirmed using Timm's stain and DiI tracing that there is minor

rearrangement of the mossy fiber pathway in mature slice cultures. Ectopically placed neurons within the hilus of mature cultured hippocampal-entorhinal cortex slices were shown to occur as a consequence of increased serum concentrations (25 %) in culture and were shown to occur along with changes in the layered organization of mature hippocampal slices, which could contribute to the hyperexcitability of those mature slice cultures after high-frequency stimulation.

The activation of astrocytes in cultured mature slices was observed in a small portion of slices cultured in high serum concentrations, and could also be a potential cause of L-LTP failure. Horse sera are known to be a significant source for glutamate, supplying glutamate at concentrations sufficient to kill primary cultured hippocampal neurons by excitotoxicity (Ye and Sontheimer, 1998; Chen et al., 2000). In slice cultures, the entire cellular network of microglia is thought to protect hippocampal neurons from excitotoxic cell death induced by glutamate. Astrocytes are known to buffer high glutamate concentrations and protect hippocampal neurons from excitotoxic cell death (Yudkoff et al., 1993). However, when astrocytes become activated their ability to protect hippocampal neurons is lost, and they can in fact contribute to neuronal death (Kingham et al., 1999; Tanabe et al., 1999; Bal-Price and Brown, 2001). Suggested mechanisms by which activated glia cells damage neurons in culture are by the release of glutamate (Barger and Basile, 2001), reactive oxygen species (Beckman et al., 1994), nitric oxide (Hu et al., 1997; Bal-Price and Brown 2001), and/or the inflammatory cytokines TNFα and IL-1β (Silver, 1994; Chao et al., 1995; Viviani et al., 1998), all of which have been shown to alter the induction and or maintenance of L-LTP (Bellinger et al., 1993; Zorumski and Izumi 1993; Cunningham et al., 1996; Schneider et al., 1998; Tancredi et al., 1992; Vereker et al., 2001). Inflammatory cytokines have also been shown to reduce the fEPSP slope and amplitude before the tetanus (Bellinger et al., 1993). I show in a small portion of mature hippocampal cultures that astrocytes became reactive indicated

by a characteristic change in morphology as well as the expression of CSPGs (Fig. 11d). In addition, the number of astrocytes significantly increased in culture, which was accompanied by a loss of their even distribution within the hippocampal layers (Fig. 11b). Astrocyte proliferation and activation is an effect of tissue culture on hippocampal tissue, and speculated to be induced by serum (Chen et al., 2000; Howard, 2000). Reactive astrocytes in cell cultures could possibly influence LTP in mature cultured hippocampal slices through the secretion of several molecules that have been shown to alter LTP directly. LTP could also, in theory, be affected indirectly by the loss of hippocampal neurons from excitotoxic cell death after the accumulation of serum born glutamate, as a consequence of the cessation of astrocyte support. Irrespective of whether serum concentration activated astrocytes or had a more direct effect on the organization of the hippocampal circuit, it was important to identify high serum concentrations as correlated with the failure of LTP and to use only slices cultured in low serum in subsequent electrphysiological experiments.

3.2.3. Mature Hippocampal-Entorhinal Cultures: a Valuable Tool in the Study of Synaptic Plasticity.

Mature hippocampal-entorhinal cultures are an excellent advance in the study of synaptic plasticity allowing one to overcome some of the experimental limitations in previous models, and providing an additional tool in the field of LTP research. They allow for experimental manipulation that is not possible in vivo, overcome the time restraint of acute hippocampal slices, and allow scientists to study LTP at the mature synapse in experiments that are better suited for the use of cultured tissue. I have shown that long-lasting LTP can be induced in cultured hippocampal-entorhinal cortex slices from mature (P25-30) rats. However, it remains to be investigated whether the prolonged maintenance of LTP in mature cultured slices is indeed characterized by the requirement

of protein synthesis, as in the intact animal and in acute hippocampal slices. One hint that cultures may show similar requirements comes from the characterization of CREB phosphorylation during long-lasting LTP in these slices.

3.3. Single-Cell Analysis of Activity-Dependent CREB Phosphorylation Following the Induction of Long-Lasting LTP in Area CA1 of Mature Hippocampal-Entorhinal Cortex Slices.

The characterization of LTP in cultured mature slices was of great significance in order to validate their use for further experiments investigating specific mechanisms involved in LTP at the mature synapse, specifically the role of the nuclear transcription factor CREB in the maintenance of long-lasting LTP. It is hypothesized that sustained activation of the nuclear transcription factor CREB is needed to maintain long-term LTP. The hypothesis was tested in mature hippocampal-entorhinal cortex slices (P25-30) by quantifying changes in CREB phosphorylation for individual CA1-pyramidal neurons during the maintenance of L-LTP using confocal microscopy.

3.3.1. Forskolin Bath Application Induces Uniform CREB Phosphorylation.

Changes in CREB phosphorylation were measured by immunohistochemically double-labeling slices with both an anti-phosphorylated CREB antibody (pCREB; specific for Ser 133 phosphorylation) and a phosphorylation state-independent anti-CREB antibody (total CREB). The two primary antibodies were visualized with immunofluorescent fluorophores and the staining intensity (mean fluorescent amplitude calculated from the integrated pixel intensity) for each antibody was measured in selected cells using confocal imaging. From the unadjusted fluorescence signal it is difficult to interpret the staining intensity of nuclear phosphorylated CREB in each cell from sections of fixated hippocampal tissue (Fig. 15a). However, by creating a composite overlay image of the

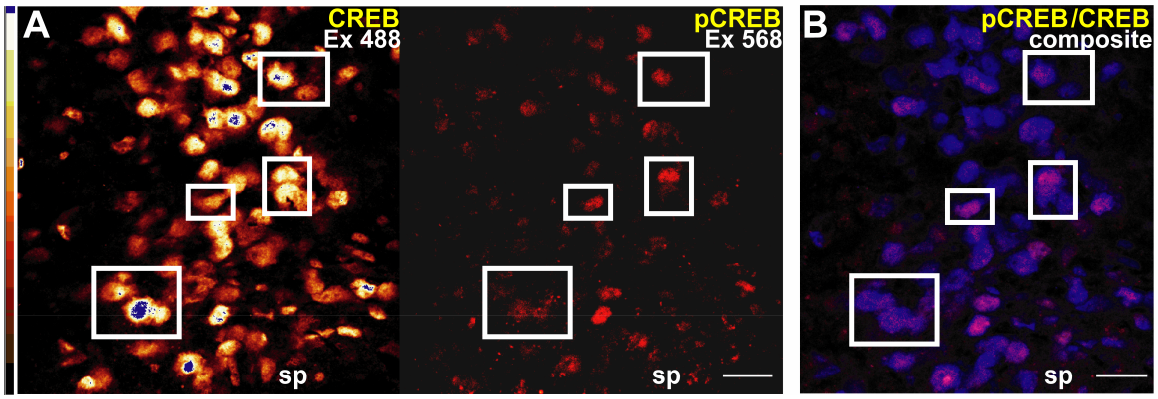

Figure 15. A composite overlay fluorescence image was created using confocal imaging to visually reflect the calculated cellular pCREB/CREB ratio. (A) Representative fluorescent images from the mid-CA1 area of a hippocampal section, following LTP. Separate images of the same labeled pyramidal neurons were simultaneously produced using confocal microscopy from both the 488 nm and 568 nm emission channels to visualize cell nuclei labeled with both a phosphorylation state-independent anti-CREB antibody and an anti-phosphorylated CREB antibody (pCREB; specific for Ser 133 phosphorylation). The value of the mean fluorescent intensity for each cell (calculated from the integrated pixel intensity) is portrayed as a color value that can be interpreted on the far-left scale bar, with *blue* reflecting the highest values. (B) A composite overlay image was created from both recording channels, using Leica imaging software, to visually portray the numerically calculated immunofluorescent pCREB/CREB ratio for each neuron. Images created from the 488 nm channel were reassigned to shades of *blue*, and the 568 nm channel *red* in order to create a visible contrast when superimposed. *White* boxes enclose the same pyramidal neurons shown in (A) and (B) for reference. High levels of pCREB immunofluorescence do not necessarily reflect a large cellular level of CREB phosphorylation. By creating a visual pCREB/CREB image the localization and extent of CREB phosphorylation can easily be observed in each cell nuclei. Scale bars shown in *A* and *B*: 20 μm. sp, stratum pyramidale.

(mean) fluorescent amplitude of labeled pCREB and CREB immunostaining, the pCREB/CREB ratio for each individual cell can be calculated, and the level of CREB phosphorylation can also be more easily interpreted visually (Fig. 15b). Such a ratio more accurately reflects relative CREB phosphorylation and allows one to compare values between experiments, which is normally prevented by the high variability of the staining procedures. Accordingly, the use of CREB or pCREB antibodies alone would fail to provide information regarding the amount of nuclear CREB phosphorylation. Changes in CREB phosphorylation in an intact hippocampal slice following the induction and

maintenance of long-lasting LTP have previously not been quantitated at a single-cell resolution, and it was first tested whether different levels of pCREB/CREB could be reliably detected by this method in a pathway known to elicit CREB phosphorylation.

CREB phosphorylation at a single-cell resolution was therefore first measured by pharmacological activation of a second messenger pathway known to phosphorylate CREB. Bath application of forskolin, an activator of the PKA cascade, has been shown to result in an increase in CREB phosphorylation (Kanterewicz et al., 2000). Analysis of forskolin-induced changes in CREB phosphorylation was performed on mature hippocampal-entorhinal cortex slices after 12 DIV. Slices were incubated in forskolin (20 μm) for various time periods (0, 5, 30, 60, 120, and 240 min; n=6 for each group), after which they were removed and immediately fixated for immunohistochemical investigation. Forskolin induced increases in cAMP resulted in an increase of the pCREB/CREB immunofluorescence ratio in cells of the entire hippocampal principal neuron population (CA1 mean cellular pCREB/CREB ratio, control, 0.116 ± 0.001, n=6; 2 h forskolin bath, 0.51 ± 0.03, n=6; Fig. 16; CA3 and dentate data not shown). The small variation in pCREB/CREB values between cells shows that CREB is activated rather uniformly.

3.3.2. LTP-Inducing Stimulation is Required for Postsynaptic Nuclear CREB Phosphorylation in Area CA1.

After validating that the ratio imaging detected the extent of nuclear CREB phosphorylation at a single-cell resolution, it was tested whether LTP induction or, as a control, any of the manipulations required for long-term extracellular recording experiments resulted in CREB phosphorylation. Changes in the pCREB/CREB ratio were assessed in slices left undisturbed in the recording chamber, slices that received continuous low-frequency stimulation (control stimulation), as well as slices in which LTP

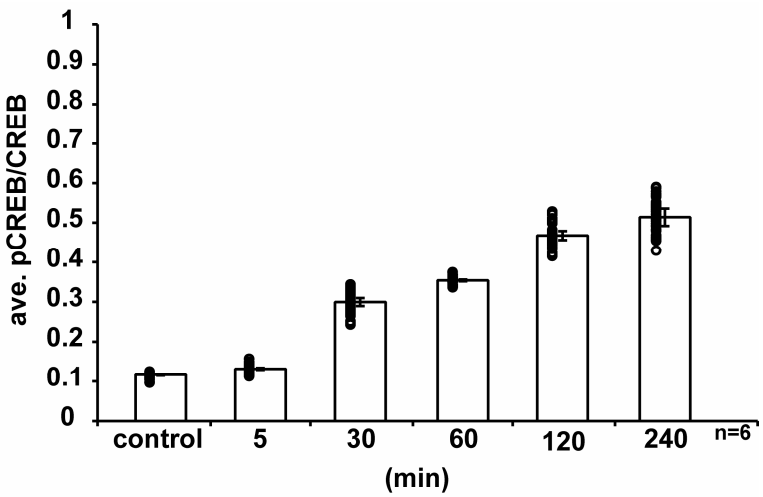

Figure 16. Forskolin-induced increases in phosphorylated CREB. CREB phosphorylation was quantitated at a cellular resolution following the induction of the PKA pathway, which is known to elicit CREB activation. A ratio of phosphorylated CREB to CREB immunofluorescence was calculated for each cell using confocal microscopy to determine the portion of phosphorylated transcription factor CREB in individual neurons. Analysis of forskolin-induced changes in CREB phosphorylation was performed on mature hippocampal-entorhinal cortex slices cultured 12 DIV, which were then incubated in either the presence or absence of 20 μm forskolin. Data represent the mean cellular pCREB/CREB ratio for CA1 pyramidal cells after slices were incubated in forskolin for various time periods (0, 5, 30, 60, 120, and 240 min; n=6 slices for each group). An increase in cAMP by forskolin resulted in a persistent and uniform increase in the average mean cellular pCREB/CREB immunofluorescence ratio, reported as the mean ± SEM. The rather uniform increase in CREB phosphorylation at the cellular level is reflected in the small variation in pCREB/CREB values between cells. Circles (o) represent the individual cellular pCREB/CREB ratios.

was induced by high-frequency stimulation in the presence or absence of a competitive antagonist of the NMDA receptor site.

Control Slices. Mature hippocampal-entorhinal cortex slices (10-15 DIV) were transferred to a carbogen-interface recording chamber and subjected to the same handling procedures and recording chamber conditions used in LTP experiments (Fig. 17). Slices were left undisturbed in the chamber for 5 h, after which they were immediately fixated for immunohistochemical investigation (n=4). Handling, chamber conditions, and extended incubation in the slice chamber did not result in a

pCREB/CREB immunofluorescence ratio that was different between pyramidal cells in CA1, pyramidal cells in CA3, or dentate granule cells. The level of CREB phosphorylation in untreated slices was uniform between hippocampal regions (mean cellular pCREB/CREB ratio at 5 h in area-CA1, 0.32 ± 0.01; area-CA3, 0.33 ± 0.02; dentate gyrus, 0.33 ± 0.01; n=4). The differences seen between individual cells also remained small as shown by the low level of variance for each subregion. It should be noted that there is a uniform increase in CREB phosphorylation for all principal hippocampal neurons following their removal from the incubator as a small increase in the mean cellular pCREB/CREB ratio is seen for control slices transferred to the recording chamber (CA1 mean cellular pCREB/CREB ratio in chamber controls, 0.32 ± 0.01), compared to slices taken fresh from culture (control CA1 mean cellular pCREB/CREB ratio for incubator controls, 0.12 ± 0.002, n=6, see Fig. 16). A chamber control slice was therefore used along with each LTP recording to directly compare each experimental condition to its own chamber control.

Low-Frequency Stimulation. In addition, it was tested whether continued low-frequency stimulation of the Schaffer collaterals could activate CREB in CA1 pyramidal cells. Again, to exclude that routine recording conditions further increased cellular CREB phosphorylation. Slices were stimulated with low-frequency control stimulation for 5 h (i.e., the length of an entire LTP experiment), and then fixated for immunohistochemical analysis. It was shown that sustained action potential firing did not significantly increase CREB phosphorylation beyond chamber control levels (chamber control CA1 mean cellular pCREB/CREB ratio at 5 h, 0.32 ± 0.01; baseline recording CA1 mean cellular pCREB/CREB ratio at 5 h, 0.30 ± 0.01, n=4). In addition, CREB phosphorylation in CA1 pyramidal cells also remained rather uniform between cells, as shown by the small variance between the pCREB/CREB ratios of individual neurons (Fig. 17). Hippocampal neurons in the CA3 area and dentate also remained at control CREB phosphorylation

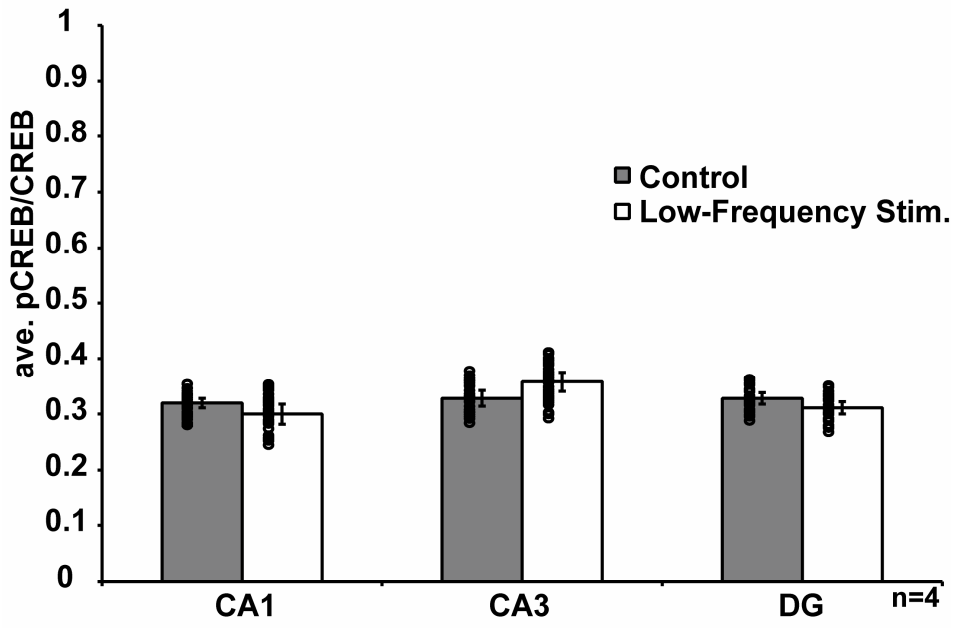

Figure 17. Low-frequency stimulation did not result in significantly increased phosphorylated CREB compared to control slices that were incubated simultaneously and left undisturbed. It was tested whether the conditions required for long-term extracellular recording experiments resulted in CREB phosphorylation. Control slices were left in the recording chamber undisturbed for 5 h, after which cellular pCREB/CREB ratios were quantitated in each region (n=4 slices). The second group received continuous low-frequency stimulation at the Schaffer collateral fibers to control for a standard recording that lasted as long as a typical LTP experiment, 5 h (n=4 slices). Handling and chamber conditions resulted in a small uniform increase in the pCREB/CREB signal, and the extended recording duration did not further increase CREB phosphorylation, which remained equal in hippocampal principal neurons of all regions. Averaged mean cellular pCREB/CREB ratios for each region are reported as the mean ± SEM. Circles (o) represent the pCREB/CREB ratios for individual cells. *DG*, dentate gyrus.

levels following low-frequency stimulation of the Schaffer collateral fibers in the CA1 area (CA3 baseline mean cellular pCREB/CREB ratio at 5 h, 0.35 ± 0.02; dentate, 0.31 ± 0.01; n=4).

High-Frequency Stimulation. After having established that CREB activation was low and uniform in response to the experimental paradigm used for LTP recordings, CREB phosphorylation was assessed for single cells following high-frequency stimulation (100Hz) of the Schaffer collateral input to the CA1 pyramidal neurons in cultured mature

hippocampal-entorhinal cortex slices (10-15 DIV). Two slices were placed within the recording chamber for each LTP experiment; one was left undisturbed to serve as an experimental control, whereas the other received the high-frequency stimulation that induced long-lasting LTP in mature cultured slices (2 trains at 100 pulses of 100 Hz, 10 min intertrain interval). Long-lasting LTP was recorded for 4 h following a 1 h baseline (n=6) after which electrodes were removed and all slices were immediately fixated for immunohistochemical analysis.

High-frequency stimulation (100Hz) of the CA1 area resulted in a potentiation of the fEPSP to 165.35 ± 13.73 % and a significant 2-fold increase of the pCREB/CREB ratio at 4 h after tetanization (Fig. 18). The increase in CREB phosphorylation was observed for CA1 pyramidal neurons (CA1 mean cellular pCREB/CREB ratio after 4 h LTP, 0.73 ± 0.06; control CA1 neurons, 0.34 ± 0.01; n=6 slices, $P < 0.05$), whereas CA3 pyramidal and dentate granule neurons remained at chamber baseline levels (CA3 mean cellular pCREB/CREB ratio after 4 h LTP, 0.40 ± 0.02; dentate, 0.35 ± 0.01; n=6 slices) indicating that antidromic stimulation of CA3 pyramidal neurons was not sufficient to increase nuclear CREB phosphorylation (Fig. 18c). Also specific for CA1 neurons following tetanization was a marked variability of CREB phosphorylation between adjacent cells as shown by the high variance in the pCREB/CREB ratio for CA1 pyramidal neurons following the 4 h maintenance of LTP (Fig. 18c). The varied level of CREB phosphorylation between CA1 pyramidal cells following long-lasting LTP is also apparent in the confocal images from slices with LTP (Fig. 19a). The high variation in the proportion of activated CREB in individual pyramidal neurons following synaptic input is in contrast to the uniform levels after long-term baseline recordings and after forskolin bath application (Fig. 19b, c). These data suggest that CREB phosphorylation is specifically involved in hippocampal plasticity, requires high-frequency synaptic input, and is enhanced to varying degrees specifically in postsynaptic neurons.

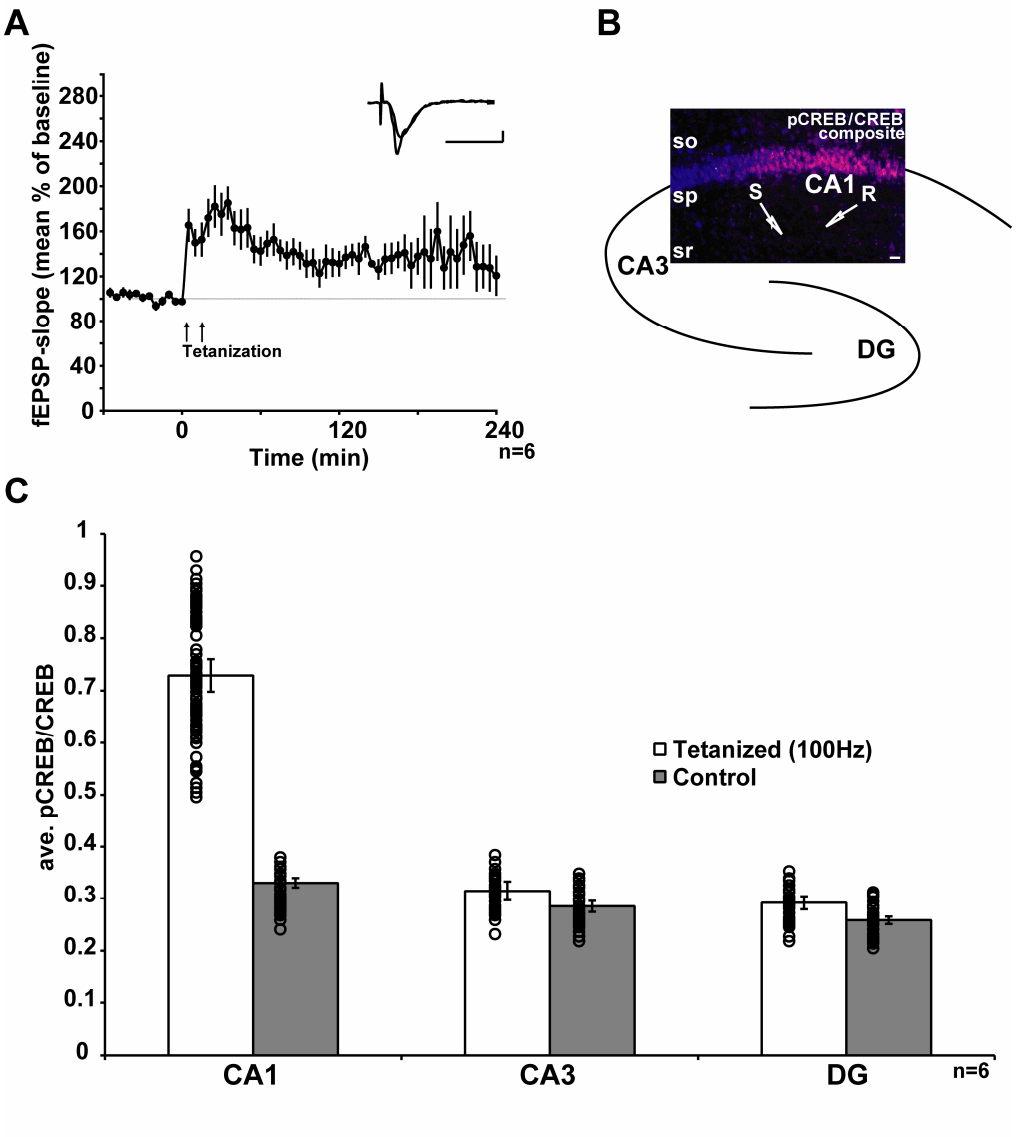

Figure 18. Individual postsynaptic CA1 pyramidal neurons show variable increases in CREB phosphorylation specifically in association with L-LTP. (A) High-frequency tetanization (HFS; 2 trains of 100 pulses at 100 Hz, 10 min intertrain interval) induced long-lasting LTP (4 h) in the CA1 area. *Insert in top right*: Superimposed representative field potentials taken 10 min before and 120 min after HFS. (B) An overlay composite pCREB/CREB image (pCREB, *red;* CREB, *blue*) created by confocal imaging at low magnification of immunostained CA1-pyramidal neurons following the induction and maintenance of 4 h LTP. CREB phosphorylation is specifically seen in postsynaptic neurons following LTP-inducing stimuli. Positions of the stimulation (S) and recording (R) electrodes are indicated by *white* arrows. The image is oriented proportionally within the illustrated hippocampal cell layers. (C) HFS of the CA1 area in mature cultured slices (n=6) resulted in a significant 2-fold increase of the pCREB/CREB ratio ($P < 0.05$) after the 4 h maintenance of long-lasting LTP. PCREB/CREB ratios were calculated for individual hippocampal neurons in area CA1, CA3, and dentate gyrus. A significant increase in CREB phosphorylation was specific for the postsynaptic CA1 pyramidal neurons, whereas CA3 pyramidal and dentate granule neurons remained at baseline levels. Specific for CA1 pyramidal neurons following the induction of LTP was a marked variation in the proportion of nuclear CREB phosphorylation as shown by the high variance in the pCREB/CREB ratios for each individual neuron, shown as (o). Averaged mean cellular pCREB/CREB ratios and fEPSP slopes are reported as the mean ± SEM. Scale bar in *A*: 0.5 mV/20 ms; in *B*: 20 μm. sr, stratum radiatum; sp, stratum pyramidale; so, stratum oriens; DG, dentate gyrus.

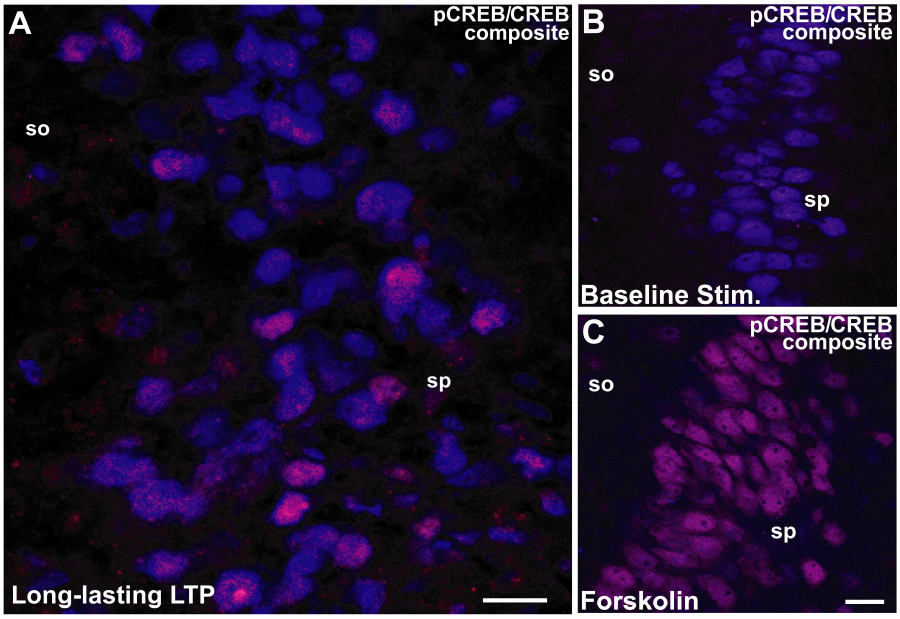

Figure 19. Differential CREB phosphorylation between individual neurons receiving synaptic input was specific for CA1 pyramidal cells only following the expression of LTP. Representative overlay composite pCREB/CREB images (pCREB, *red;* CREB, *blue*) were created from fluorescent images acquired by confocal microscopy at high magnification in the mid-CA1 area of hippocampal slice sections. Slices were immunostained following either (A) the 4 h maintenance of LTP, (B) 5 h routine baseline recordings using low-frequency stimulation, or (C) 4 h incubation in the presence of 20 μm forskolin, an activator of PKA. CREB is constitutively expressed as shown by the labeling of CA1-pyramidal neurons (*blue*). The high variability in the portion of phosphorylated CREB in adjacent pyramidal neurons following high-frequency stimulation of the synaptic input (A) is in contrast to the uniform increase in CREB phosphorylation after forskolin bath application (C) and in contrast to the lack of CREB phosphorylation following continued low-frequency stimulation (B). Scale bar in *A* and *C*: 20 μm. sp, stratum pyramidale; so, stratum oriens.

To determine whether CREB phosphorylation in mature hippocampal-entorhinal cultured slices requires NMDA receptor activation, the slices were treated with the NMDA receptor antagonist APV prior to LTP induction and the level of CREB phosphorylation was investigated 2 h after a 100 Hz stimulus (Fig. 20). When 100 Hz stimulation (i.e., 2 trains of 100 bursts at 100 Hz with a 10 min intertrain interval) was applied to the CA1 area in the presence of APV (50 μM), the potentiation was significantly reduced relative to control LTP. Moreover, APV not only prevented the

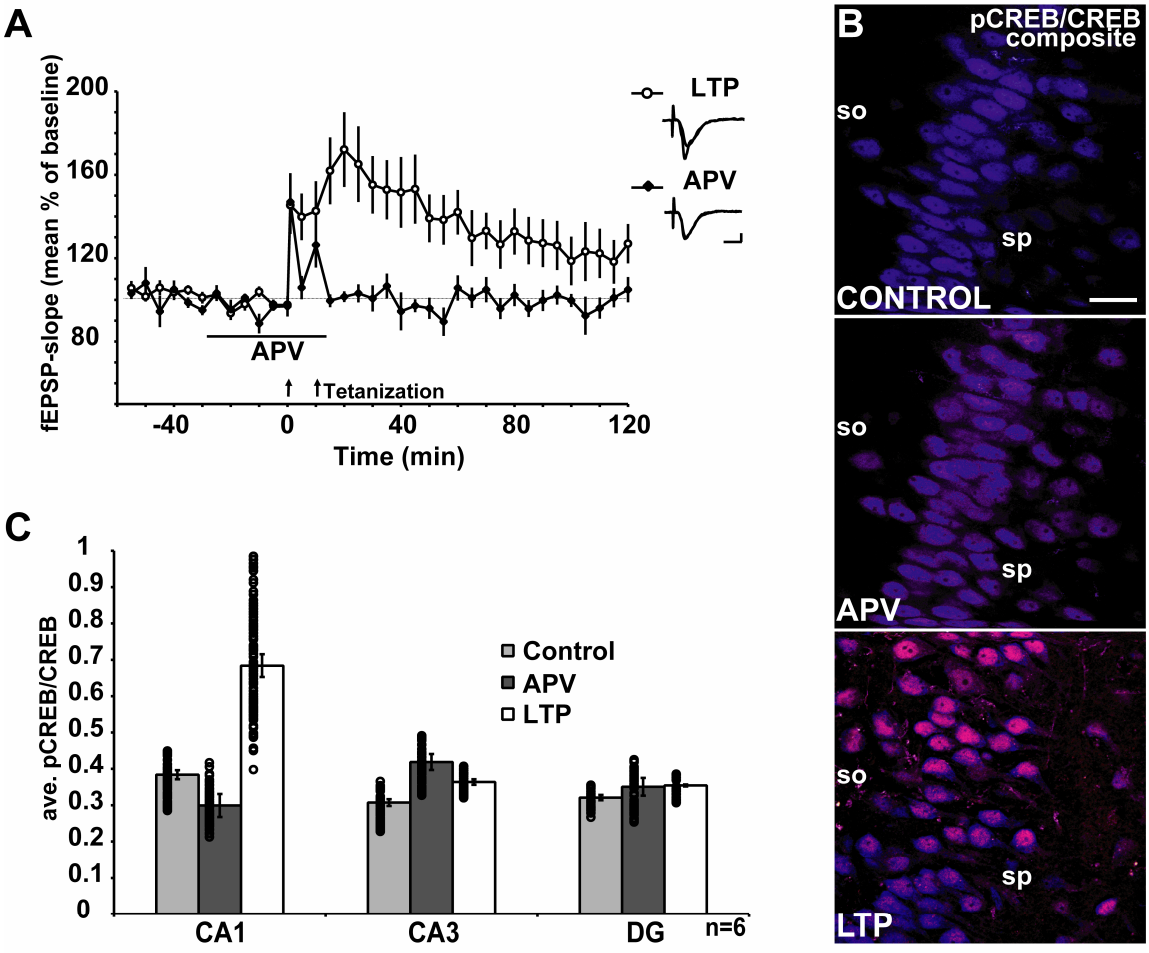

Figure 20. L-LTP and associated increases in cellular CREB phosphorylation require NMDA receptor activity. (A) L-LTP in area CA1 was blocked by 50 μM APV (♦) compared to control LTP (o) after HFS. *Right*: Superimposed representative field potentials 10 min before and 30 min after HFS of the Schaffer collaterals (electrophysiological APV data was in part presented previously, Fig. 14, section 3.1.4). (B) Representative composite pCREB/CREB images (pCREB, *red;* CREB, *blue*) of the mid-CA1 area in an untetanized control slice, and slices fixated 120 min after HFS in the presence and absence of 50 μM APV. Images show that APV also blocked the enhancement of CREB phosphorylation specific for the expression of LTP in the absence of APV. (C) Individual cellular pCREB/CREB ratios calculated in all principal hippocampal neurons of control slices, and slices fixated 120 min after HFS in the presence and absence of 50 μM APV (n=6). APV blocked the enhancement of CREB phosphorylation, which remained at control levels. Scale bars in A: 0.5 mV/10 ms; *B*: 20 μm. sp, stratum pyramidale; so, stratum oriens.

induction of LTP but also blocked the enhancement of CREB phosphorylation, which remained at control levels (CA1 mean cellular pCREB/CREB ratio after 2 h in the absence of APV, 0.68 ± 0.07, n=6; in the presence of APV, 0.30 ± 0.03, $P < 0.05$, n=6; control, 0.38 ± 0.01, n=6). Thus, long-lasting LTP and the associated increases in CREB phosphorylation in mature hippocampal-entorhinal cortex cultures require NMDA receptor activity.

3.3.3. Spatio-Temporal Dynamics of CREB Phosphorylation during Hippocampal LTP in Area CA1.

CREB phosphorylation was assessed at a single-cell resolution at different time intervals (30, 120, 240 min; n=6 each group) following LTP induction by high-frequency stimulation (100Hz) in the CA1 area of cultured mature hippocampal-entorhinal cortex slices (10-15 DIV). In addition to investigating the temporal dynamics of the average CREB phosphorylation after LTP in mature cultured slices, it was further investigated to what extent nuclear CREB phosphorylation changed throughout the neuron population activated by high-frequency stimulation, and if changes in the proportion of CREB activated at a cellular level occur during the maintenance of LTP. LTP was accompanied by a significant increase in the pCREB/CREB ratio at 30 min, which continued to increase after LTP had lasted for longer intervals (CA1 mean cellular pCREB/CREB ratio at 30 min, 0.50 ± 0.05; at 2 h, 0.60 ± 0.06; at 4 h, 0.73 ± 0.06; n=6, $P < 0.05$, Fig. 21). The sustained phosphorylation of CREB was specific for CA1 pyramidal cells, whereas CA3 pyramidal cells and dentate granule cells remained at baseline levels for the entire LTP duration (4 h time point shown in Fig. 18, additional data not shown). Moreover, the striking variation of CREB activation between CA1 neurons, after LTP induction remained throughout the duration of LTP (Fig. 21), but with the level of CREB

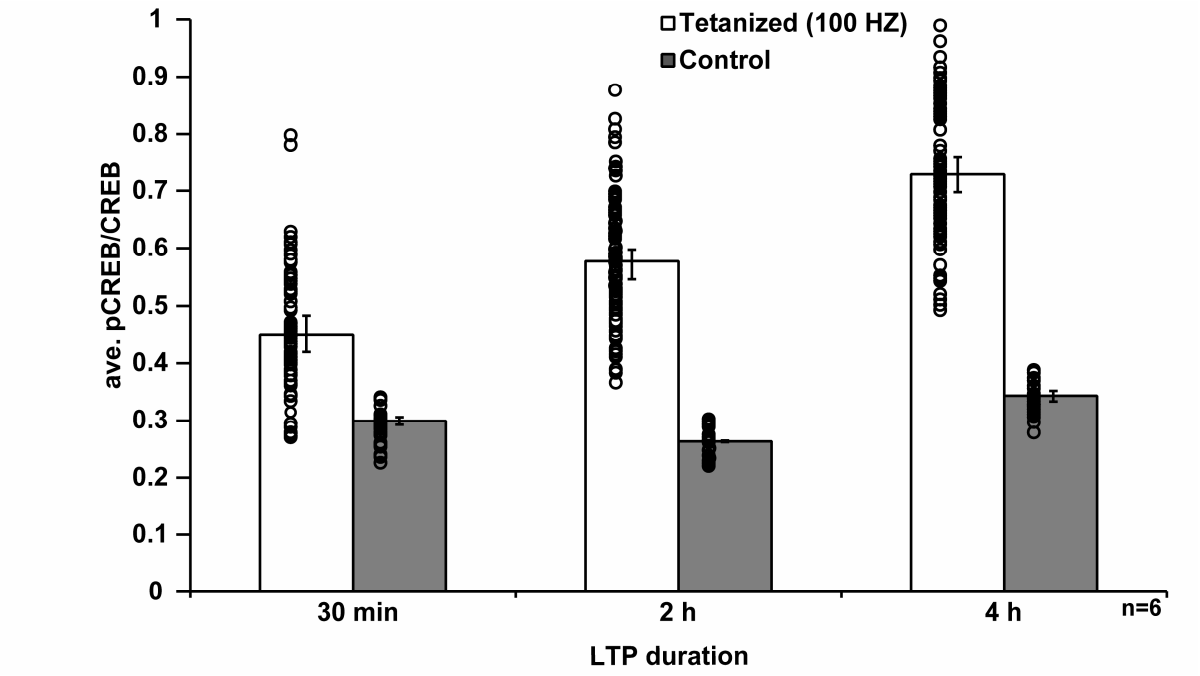

Figure 21. CREB phosphorylation increased continually in the entire population of CA1-pyramidal neurons in slices where long-lasting LTP was induced. CREB phosphorylation was assessed at a single-cell resolution following HFS (100 Hz) of the CA1 stratum radiatum, after which LTP was recorded for increasing time intervals (0, 30, 120, 240 min; n=6 each group). LTP resulted in an initial increase in the mean cellular pCREB/CREB ratio, which continued to significantly increase in parallel to the increased duration of LTP (to 4 h; $P < 0.05$). The high variability of CREB phosphorylation between individual CA1-pyramidal neurons following the induction of LTP is seen for each time point. The cellular pCREB/CREB ratios calculated for each neuron (o) are shown. The variation between pyramidal neurons remained high, but did not further increase for later time points. The averaged cellular pCREB/CREB ratios are reported as the mean ± SEM.

phosphorylation increasing in the entire population of CA1 pyramidal neurons after longer LTP durations.

3.4. Discussion of Single-Cell Analysis

The level of CREB phosphorylation was measured at a single-cell resolution in organotypic hippocampal slices from young adult rats (P25-30) at different time points throughout the duration of long-lasting LTP. CREB phosphorylation was shown to occur

in postsynaptic neurons only in response to stimuli that were sufficient to result in LTP. High-frequency stimulation (100Hz) of the CA1 area induced long-lasting LTP and a 2-fold increase compared to chamber controls of the pCREB/CREB ratio at 4 h after tetanization (see Fig. 18). This increase in CREB phosphorylation was specific for conditions that increased the fEPSP of postsynaptic CA1 pyramidal neurons (Fig. 18c), and did not occur during continued low-frequency activation (i.e., 5 h baseline recordings; see Fig. 17).

It was critical to provide adequate controls to differentiate between stimulus-specific processes and changes related to routine LTP recordings. Each experiment in this study included 2 slices from the same animal, which were cultured and transferred to the recording chamber together. One of these slices was always left undisturbed to continually provide verification that the recording environment in each trial did not significantly induce CREB phosphorylation. It should be noted that there is a uniform increase in CREB phosphorylation for all principal hippocampal neurons following their removal from tissue culture incubation (compare incubator control values from Fig. 16 to all chamber controls). However, the degree of change is of a consistent magnitude and uniform for all principal hippocampal neurons and occurs presumably due to the low basal activity of quiescent slices in culture and the subsequent activation of normal “house-keeping” functions. By using chamber controls in all LTP experiments in this study (shown in each control group, Fig. 17-21), it can be concluded that CREB was not further activated in response to the baseline stimulation used for LTP recordings in cultured mature hippocampal-entorhinal cortex slices.

The stability of mature cultured slices during routine LTP recordings (Section 3.1; Leutgeb et al., 2003) makes them an excellent model for investigating CREB activation in relation to the maintenance of long-lasting synaptic enhancement. It is crucial to choose an adequate model to investigate CREB-function during LTP for it has been

shown recently that acute hippocampal slices from genetically engineered mice with deficiencies in CREB-gene-dosage are potentially inappropriate models for such studies (Gass et al., 1998; Balschun et al., 2003). The acute hippocampal slice has been a preferred model for the investigation of LTP. However, its limited viability has raised concerns that such a model might not accurately reflect changes of CREB phosphorylation in healthy intact neural systems. It has been shown that the routine incubation of acute slices alone can result in the up-regulation of immediate early-genes, whose transcription depends on CREB activation, presumably as a result of hypoxic injury and apoptosis (Zhou et al., 1995). Moreover, CREB can be specifically phosphorylated during the activation of neuronal cell death and apoptosis (Vyas et al., 2002) as well as in the processes of neuronal survival (Finkbeiner et al., 1997; Walton and Dragunow, 2000; Mantamadiotis et al., 2002). These processes are of particular concern when correlating CREB phosphorylation with L-LTP recordings in acute slices with temporally limited viability and a greater potential for necrosis. Mature hippocampal slices on the other hand have been shown to be free of cell death and necrosis after 7 DIV (Xiang et al., 2000), and the phosphorylation of CREB has been shown to remain at stable baseline levels throughout the entire length of all L-LTP experiments in this study.

Previous investigation of the temporal dynamics of CREB phosphorylation has revealed that the multiple phases of LTP are associated with different patterns of CREB phosphorylation, which differed depending on the experimental model. Matthies et al., (1997) investigated the temporal dynamics of CREB phosphorylation in acute hippocampal slices and observed that high-frequency stimulation (100 Hz) in the CA1 area resulted in L-LTP (4 h) associated with an immediate increase in CREB phosphorylation. The phosphorylation of CREB was transient and declined after 30 min despite continued synaptic enhancement. Transient CREB phosphorylation that

disappeared by 2 h is observed in vivo only after inducing decremental E-LTP after perforant path stimulation (Schulz et al., 1999). Nondecremental L-LTP resulted in a biphasic CREB phosphorylation with an initial robust peak at 30 min and sustained phosphorylation of CREB beginning at 2 h LTP and lasting up to 24 h. The induction of long-lasting LTP in the CA1 area of mature cultured slices resulted in the sustained phosphorylation of nuclear CREB. CREB phosphorylation was first observed at 30 min after the induction of LTP, and remained after 4 h of maintenance (Fig. 21). Moreover, from calculating the individual cellular PCREB/CREB ratio for individual cells, it was further shown that increases occur throughout the entire CA1 cell population during the maintenance of long-lasting LTP. These findings coincide better with the dynamics of CREB phosphorylation described in vivo, rather than the pattern observed during L-LTP in acute slices, and suggests that the differences in the experimental models rather then the region of LTP induction resulted in the opposing results. Accordingly, only L-LTP induced in experimental models characterized by stable, intact neural connections has been shown to be associated with stable, sustained CREB phosphorylation.

In contrast to previous studies that have inferred an increase in CREB phosphorylation by the visual comparison of fluorescent images from entire neuronal populations, I have quantified the nuclear CREB phosphorylation in individual neurons during the duration of LTP to provide additional information on the dynamics of CREB activation during hippocampal long-term synaptic plasticity. CREB was phosphorylated in individual pyramidal cells as a result of the induction of long-lasting LTP in mature cultured slices (Fig. 18, 21), and it was shown for the first time that activated nuclear CREB in individual pyramidal neurons following synaptic input varied significantly between adjacent neurons. Differential CREB phosphorylation between neurons receiving synaptic input was specific for CA1 pyramidal cells only following the expression of LTP, which is reflected in the high variance of the pCREB/CREB ratio for

individual CA1 pyramidal neurons (Fig. 18c, 20, 21). This phenomenon was accompanied consistently by a minimal variance between individual neuron pCREB/CREB ratios for all experiments and cell regions other than those receiving high-frequency stimulation. The striking variation in CREB activation observed between CA1 neurons, specifically as a result of LTP induction, remained throughout the duration of long-lasting LTP while increases in CREB phosphorylation were observed throughout the entire pyramidal cell population. This pattern suggests that the neurons initially recruited by high-frequency stimulation phosphorylated CREB at different degrees in response to postsynaptic input and that increases occurred irrespective of the initial level of CREB phosphorylation. The fact that increases in the portion of nuclear CREB phosphorylation occur in a consistent pattern and magnitude during the maintenance of LTP, suggests that high-frequency stimulation results in varied CREB activation by initially inducing phosphorylation at different levels. Differences in the cellular levels of CREB phosphorylation were unique to LTP induced by tetanic stimulation in mature slices. In contrast to chemical stimulation in vitro, which drives cellular signaling systems to steady-state equilibrium, electrical stimulation models the dynamic intracellular signaling system driven by neural activity. Therefore, the differences in the cellular level of CREB phosphorylation after electrical stimuli possibly reflect differences in the number of active inputs perceived at each cell.

The variation in the portion of CREB activated in individual pyramidal neurons following synaptic activation was in contrast to the uniform control levels and to the uniform increase in CREB phosphorylation following bath application of forskolin (Fig. 19). Cellular increases of cAMP induced by forskolin bath application, which is known to activate the PKA pathway and to result in L-LTP (Scheng et al., 1990; Impey et al., 1998; Deisseroth et al., 1998; Kanterewicz et al., 2000) was shown to induce CREB phosphorylation that was uniform across all pyramidal cells and gradually increased with

continued bath application of forskolin in mature cultured slices (Fig. 16). Electrical stimulation, on the other hand, resulted in a more immediate increase in CREB phosphorylation that initially reached higher maximum pCREB/CREB levels than those induced by forskolin bath application (compare Fig. 16, 18c, and 21).

The activation of CREB in mature hippocampal-entorhinal cortex cultures requires NMDA receptor activity, as does the induction of L-LTP, for both processes were blocked when high-frequency stimulation was given in the presence of the NMDA receptor antagonist APV (Fig. 20). It is intriguing that in the signaling mechanisms controlling the phosphorylation of CREB individual neurons appear to also be required for the induction of LTP as shown by the fact that not even single cells showed increases in phosphorylation without the activation of NMDA receptors.

The signaling pathways involved in synaptic plasticity and for CREB signaling might share common steps. However, the threshold for the activation of phosphorylation may be different for each cell. The differences in the magnitude of CREB phosphorylation after tetanic stimulation could also reflect relative differences in the activation of different signaling pathways. LTP induced in mature hippocampal slices was shown to be NMDA-receptor dependent (Fig. 20). Activation of NMDA receptors can activate both CamK and PKA signaling pathways (reviewed in West et al., 2001), both of which phosphorylate CREB at different times and durations (Impey et al., 1998; Wu et al., 2001; Deisseroth and Tsien, 2002). The coupling of these pathways was shown to result in sustained CREB phosphorylation (Impey et al., 1998). These findings suggest that the kinetics of intracellular signaling pathways can each in turn convey information to the nucleus about the pattern of synaptic stimulation, such as timing or duration, and activate CREB depending on the received synaptic input pattern.

Accordingly, changes in the concentration of postsynaptic Ca^{2+} as a result of synaptic input could result in varied levels of CREB phosphorylation between CA1 cells in mature

slices. Cell specific translation of input patterns and strength to gene expression for the maintenance of enhanced plasticity is a proposed function of CREB phosphorylation. It has been shown in dorsal root ganglion neurons in vitro that CREB phosphorylation is a relatively poor indicator of stimulus pattern (Fields et al., 1997), but changes in the stimulus burst duration and the peak increase in Ca^{2+} concentration after stimuli produced differences in the degree of CREB phosphorylation. The shortest bursts produced the least increase in Ca^{2+} concentration, but the highest level of CREB phosphorylation, which could be explained by kinase, or phosphatase activity. In fact, CREB phosphorylation as a result of a weak priming stimulus has been shown to occur faster and be more sensitive to Ca^{2+} elevation (Mermelstein et al., 2001), suggesting that nuclear processing of synapse-to-nucleus signals can result in varied CREB phosphorylation. In this study cells varied in the degree of CREB phosphorylation following LTP induction by high-frequency stimulation, possibly as a result of cells receiving different numbers of active input. Differences in the location or number of synapses activated in response to the stimulus event could be conveyed to the nucleus and reflected in the varied degrees of CREB phosphorylation.

Many questions remain to be answered in terms of the influence of dendritic processing on nuclear CREB activation. Irrespective of the widely different levels of CREB phosphorylation, why did the pattern of activation not seem to diverge with increasing overall levels of CREB activation? How many active synapses are needed to trigger nuclear CREB phosphorylation, are fewer activated synapses translated to a lower level of CREB phosphorylation and transcription? To what extent does the level of dendritic processing influence the level of CREB phosphorylation in the nucleus? The dynamics of CREB activation in response to information conveyed from the synapse to the nucleus and its correlation to the mechanisms of L-LTP prove to be a complicated puzzle, and will require continued investigation.

4. Conclusions

The experiments of the present dissertation asked whether hippocampal slices from young adult rats (P25-30) could be used to investigate long-term plasticity, which may also occur during learning in adult organisms. Studying related processes in immature slices may be of limited relevance due to differences in the cellular processes of synaptic plasticity at immature synapses. The electrophysiological characteristics, in particular the parameters for long-lasting LTP induction were first established in mature cultures. The mature cultures were then used to investigate the relationship between the maintenance of long-lasting LTP and the activation of CREB by phosphorylation at the Ser 133 site. It was hypothesized that continued phosphorylation of the nuclear transcription factor CREB is needed to sustain long-lasting LTP. The principle findings of this dissertation are as follows:

1) Improved tissue culture methods and new electrophysiological protocols were required to obtain mature hippocampal slice cultures that do not only show synaptic signals, but also synaptic plasticity. Cultured hippocampal-entorhinal cortex slices from young adult rats (P25-30) retain electrophysiological stability and the capability for recording long-lasting LTP after 2-3 weeks in vitro.
2) It was shown that the temporal pattern of CREB phosphorylation in mature cultured slices matches the pattern that has previously been observed in vivo better than what was shown for acute hippocampal slices.
3) The spatio-temporal dynamics of CREB phosphorylation were investigated by analysis at a single-cell resolution. It was shown that the induction of long-lasting LTP resulted in varied amounts of CREB phosphorylation in CA1 pyramidal neurons that as a population expressed an enhancement in synaptic strength. This observation was specific for electrical stimulation that resulted in LTP, and limited to

the cell population that also underwent a change in synaptic strength. In addition, the proportion of phosphorylated nuclear CREB during the maintenance of L-LTP continued to increase in the entire CA1 pyramidal cell population irrespective of the initial cellular levels of phosphorylation.

The mechanisms that turn on gene expression subsequent to learning events leading to long-term synaptic change and memory consolidation are uncertain. If in fact the late phases of LTP are responsible for memory formation by leading to long-lasting changes in synaptic strength and structure, then understanding the mechanisms and events by which this occurs is extremely important, but technically very difficult. I have shown that molecular and electrophysiological plasticity can be studied in parallel in mature cultured tissue. Long-lasting electrophysiological and technically advanced in vitro experiments using adult tissue are possible with the development of mature hippocampal-entorhinal cultures. This experimental freedom can serve as an invaluable tool for further investigating the events of synapse-to-nucleur signaling responsible for long-term synaptic plasticity.

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APPENDICES

I. ZUSAMMENFASSUNG DER DISSERTATION

Die Mechanismen, die zur Protein- und Genexpression nach Lernen und in der Folge zu lang anhaltenden synaptischen Veränderungen sowie der Gedächtniskonsolidierung führen, sind noch weitgehend unbekannt. Falls die späten Phasen der Langzeitpotenzierung (LTP, engl.: long-term potentiation) zu dauernden strukturellen synaptischen Veränderungen und daher zur Langzeitgedächtnisbildung führen, ist das Verständnis dieser Prozesse wichtig, aber auch methodisch schwierig zu untersuchen. Zusätzlich zu akut präparierten Hippokampusschnitten von adulten Organismen werden daher auch hippokampale Zell- und Gewebekulturen, die bisher von embryonalen und juvenilen Organismen gewonnen wurden, für Untersuchungen der LTP verwendet. Kulturen von juvenilem Gewebe zeigen allerdings nicht nur in der anatomischen und zellulären Entwicklung Unterschiede zur ontogenetischen Entwicklung des Tieres, sondern auch im Hinblick auf die zur synaptischen Plastizität führenden Mechanismen. Wichtig ist daher die Suche nach einem Forschungsmodell, das auch adultes Gewebe in vitro Experimenten zugänglich zu macht. Daher war es Ziel der vorliegenden Dissertation, hippokampales Gewebe mit bereits ausgebildeten synaptischen Verbindungen zu kultivieren und organotypische Schnittkulturen von jung-adulten Ratten als zusätzliches Modell zum Studium der LTP zu etablieren. Für diese Präparation wurde Gewebe von 25 bis 30 Tage alten Tieren verwendet, da die Entwicklung des Gehirns zu diesem Zeitpunkt größtenteils abgeschlossen ist. Außerdem wurden die elektrophysiologischen Eigenschaften der Schnittkulturen und speziell die synaptische Plastizität untersucht.

In der vorliegenden Arbeit wurden zunächst die Effekte verschiedener Präparationsmethoden und Kultivierungsbedingungen auf die morphologischen und funktionellen Eigenschaften der kultivierten Hippokampusschnitte von jung-adulten Ratten beschrieben. Mit Hilfe elektrophysiologischer und immunohistochemischer Methoden wurde festgestellt, ob nach der Kultivierung physiologisch aktive Synapsen vorhanden sind und ob die Einführung optimierter Methoden die elektrophysiologischen Eigenschaften von hippokampalen Schnitten verbesserte. Mit den modifizierten Protokollen war es möglich auch nach 2-wöchigem Kultivieren der Hirnschnitte funktionell intakte Synapsen zwischen Tractus perforans und Granularzellen im Gyrus dentatus, Moosfasern und CA3 Zellen als auch an den Verbindungen zwischen Schafferschen Kollateralen und CA1 Pyramidenzellen zu gewährleisten. Die elektrophysiologischen Eigenschaften der adulten Schnittkulturen wurden wiederholt geprüft, insbesondere um Stimulationssequenzen für die Induktion einer mehrstündigen LTP in der CA1 Region zu finden. Mit den hier entwickelten Protokollen konnte die LTP in CA1, wie in akut präparierten Schnitten, erstmalig auch in adulten Schnittkulturen verlässlich nach extrazellulärer Stimulation induziert werden. Ihre Induktion war NMDA-Rezeptor-abhängig und ihre Aufrechterhaltung lang andauernd (> 4 Stunden). In den neu entwickelten hippokampal-entorhinalen Hirnschnittkulturen von jung-adulten Ratten bleiben daher die für die Langzeitpotenzierung erforderlichen Eigenschaften erhalten, und die Kulturen erlauben in vitro Untersuchungen der Plastizität neuronaler Verbindungen, deren ontogenetische Entwicklung vor der Präparation weitgehend abgeschlossen war.

Die Phosphorylierung des Transkriptionsfaktors CREB (engl.: cAMP response element binding protein) wird sowohl für die synaptische Plastizität als auch für Gedächtnisprozesse als wesentlich erachtet. Die hippokampal-entorhinalen

Schnittkulturen von adultem Gewebe wurden nun verwendet, um die Beziehung zwischen der lang anhaltenden LTP und der zeitlichen und räumlichen Dynamik der CREB Phosphorylierung zu visualisieren. Die Dynamik der CREB-Aktivierung wurde durch die Verwendung von immunhistologischen Methoden und konfokalen bildgebenden Verfahren erstmalig auch mit zellulärer Auflösung beobachtet. Die Methode wurde zunächst nach Erhöhung der cAMP Konzentration durch die Zugabe von Forskolin getestet. Eine Normalisierung und Quantifizierung der relativen CREB-Phosphorylierung war durch das Bestimmen des Verhältnisses zwischen phosphoryliertem CREB (pCREB) und nicht phosphoryliertem CREB möglich, und nach der Applikation von Forskolin wurde eine stetige Erhöhung der pCREB/CREB Immunfluoreszenz in der gesamten Population der hippokampalen Pyramidenzellen beobachtet. Nach elektrischer Stimulation des CA1 Areals mit einer Frequenz von 100 Hz (Tetanisierung) konnte eine lang anhaltende LTP induziert werden und ebenfalls eine signifikante Erhöhung des Anteils von pCREB detektiert werden. Zudem stieg der durchschnittliche pCREB/CREB Wert während der Aufrechterhaltung der lang andauernden LTP weiter an. Eine erhöhte CREB Phosphorylierung wurde nach der LTP Induktion nur im CA1 Gebiet beobachtet, und die Werte zeigten eine hohe Variabilität zwischen einzelnen, auch benachbarten CA1 Neuronen. Im Gegensatz zu den Ergebnissen nach Forskolin Applikation wurden nach der LTP Induktion keine Unterschiede zu Kontrollwerten für Messungen im CA3 Areal und im Gyrus dentatus gefunden. Die für das CA1 Areal spezifische CREB-Phosphorylierung weißt darauf hin, dass weder die antidrome Stimulation noch spezifische Messbedingungen für die Induktion der CREB-Phosphorylierung nach der Tetanisierung ausreichend waren. Es wurde außerdem kein Anstieg des pCREB/CREB-Wertes gemessen, wenn die Tetanisierung während der Applikation von APV erfolgte und nur eine posttetanische Potenzierung induziert wurde. Sowohl die Aktivierung von CREB als auch die LTP

Induktion in Schnittkulturen adulter Ratten sind daher NMDA-Rezeptor-abhängig. Durch die hier verwendeten Methoden konnte erstmalig gezeigt werden, dass die Induktion der LTP zu unterschiedlicher CREB Aktivierung in einzelnen Pyramidenzellen führt und während der gesamten Dauer der LTP in der CA1 Zellpopulation weiter zunimmt. Die Hypothese, dass die CREB-Aktivierung eine wichtige Rolle während der Expression und Aufrechterhaltung von lang andauernder LTP spielt, wird durch die vorliegenden Resultate weitgehend unterstützt.

In der vorliegenden Dissertation habe ich gezeigt, dass die molekularen und elektrophysiologischen Eigenschaften der Schnittkulturen denen jung-adulter Organismen entsprechen und in vitro untersucht werden können. Mit der Entwicklung der hippokampal-entorhinalen Schnittkulturen von jung-adultem Gewebe ist der Einsatz von Techniken möglich, die in anderen Modellen der Langzeitplastizität nur begrenzt anwendbar sind. Diese experimentellen Möglichkeiten können für weitere Untersuchungen von großer Bedeutung sein, insbesondere im Hinblick auf nun möglich werdende Untersuchungen der für die Langzeitplastizität wesentlichen Signaltransduktionswege zwischen der Synapse und dem Nukleus.

II. SELBSTÄNDIGKEITSERKLÄRUNG

Erklärung

Hiermit erkläre ich. dass ich die von mir eingereichte Dissertation mit dem Thema

> Synaptic Plasticity in Mature Cultured Hippocampal-Entorhinal Cortex Slices: Activity-Dependent Regulation of cAMP Response-Element Binding Protein

selbständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Trondheim, Norwegen, 14.10.2003

Master of Science-Biologie. Jill K. Leutgeb

III. PUBLICATIONS

Leutgeb JK, Frey JU, Behnisch T (2003) Single-cell analysis of activity-dependent CREB phosphorylation following the induction of long-lasting LTP in area CA1 of mature rat hippocampal-organotypic slices. (Submitted).

Leutgeb JK, Frey JU, Behnisch T (2003). LTP in cultured hippocampal-entorhinal cortex slices from young adult (P25-30) rats. J Neurosci Methods 130(1):19-32.

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Leutgeb J, Frey JU, and Behnisch T (2002) LTP in mature rat organotypic slices results in cell-specific CREB activation. Soc. Neurosci. Abstr. 151.6.

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IV. CURRICULUM VITAE

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EDUCATION

2000-present Doctoral Student
Department of Neurophysiology, Leibniz Institute for Neurobiology and Otto-von-Guericke Universität, Magdeburg, Germany
Full scholarship, Leibniz Institute for Neurobiology, Magdeburg, Germany
Dissertation Title: "*Synaptic Plasticity in Mature Cultured Hippocampal-Entorhinal Cortex Slices: Activity Dependent Regulation of cAMP Response-Element Binding Protein"*

Aug 2000 Master of Science
1998-2000 Department of Oncological Sciences-Graduate Program in Neuroscience, University of Utah
Full Scholarship, Neuroscience Program, College of Medicine, University of Utah
Thesis title: "*Growth Factor Expanded-Glial Restricted Precursor Cells: Support of Neurons by Derived Astrocytes"*

Dec 1998 Bachelor of Science
1993-1998 Department of Biology, University of Utah
Major degree: Biology, Minor degree: Chemistry
Senior thesis title: "*Glutamatergic Splice Variants 'Flip and Flop' in the Aged Mouse Brain".*

RESEARCH PROJECTS

2000-2002 Research Assistant, Advisor: Prof. Dr. Julietta Uta Frey
Dissertation Project: Synaptic plasticity in cultured mature hippocampal-entorhinal cortex slices. Activity-dependent changes in CREB (cAMP response element) during L-LTP in young adult rat (P25-30) cultured

slices. *Methods: Tissue culture, extracellular field potential recordings, immunohistochemistry, protein chemistry, microscopy (2-photon, confocal), development of single-cell quantitative fluorescent imaging.*

1999-2000 Research Assistant, Advisor: Dr. Mark Noble
Thesis Project: Changes in neurite outgrowth and cell adhesive properties of astrocytes derived from growth factor expanded-glial restricted precursor cells: therapeutic use of glial precursor cells. *Methods: Tissue culture (primary cell, stem cell, neuronal and glial precursor cell), protein chemistry, molecular biology, immunocytochemistry, confocal microscopy.*

Summer 1999 Research Assistant, Advisor: Dr. Mark Noble
Project: Neuronal regeneration following CNS glial scarring, precursor cell biology, and genetic engineering of cell substrates to enhance neuronal regeneration. *Methods: Molecular biology (DNA sub-cloning, RT-PCR, RNA isolation, cDNA library, design and construction of bacterial and viral plasmids, transfection), immunohistochemistry, tissue culture, microscopy, protein chemistry.*

Spring 1999 Rotation Student, Advisor: Dr. Stanley B. Kater
Project: Role of CREB (cAMP response element) in glial Ca^{2+} waves. *Methods: Ca^{2+} imaging, tissue culture, immunohistochemisty*.

Winter 1998 Rotation Student, Advisor : Dr. Robert Marc
Project: Mapping the ionotropic glutamatergic drive histories of amacrine cell layer neurons reported by 1-Amino-4-Guanidobutane (AGB) in vivo. *Methods: Tissue culture, immunohistochemistry, computer analysis (GSS mapping software).*

Fall 1998 Rotation Student, Advisor: Dr. Mark Noble
Project: Glial progenitor cell biology: Nicotine receptor involvement during glial progenitor cell development. *Methods: Ca^{2+} imaging, single-cell RT-PCR, tissue culture.*

Summer 1998 Rotation Student, Advisor: Dr. Scott Rogers
Project: Cytokine-mediated neuroprotection in response to alcohol. *Methods: Molecular biology (RT-PCR, RNA isolation, subcloning, transfection), protein chemistry (western blot, immunoprecipitation), tissue culture, cobalt staining, immunocytochemistry, microscopy.*

June 1998 Bachelor of Science Senior Project, Advisor: Dr. Scott Rogers
Title: “Glutamatergic Splice Variants ‘Flip and Flop’ in the Aged Mouse Brain”. *Methods: Sub-cloning, RT-PCR, RNA isolation, immunohistochemistry, microscopy.*

1996-1998 Laboratory Technician, Advisor: Dr. Scott Rogers
Project: Structure and function of neuronal nicotinic receptors and glutamate receptors: Molecular mechanisms of neuro-immune interactions. Changes in the expression of these receptor systems were examined in the aging brain as well as in response to inflammatory

responses using various molecular biology and protein chemistry techniques.

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