Homeostatic regulation of long-term potentiation

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Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Beihilfe angefertigt ist.

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1. Summary

In developing neuronal circuits, homeostatic regulation of global synaptic strength can operate over a timeframe of several days (Turrigiano et al., 1998). However, also in the adult nervous system, protection against runaway synaptic strengthening might be necessary, especially in highly plastic brain structures such as the hippocampus. If too many synapses of a single neuron are potentiated, a neuron might be at risk of overexcitation; and in addition, this might reduce the information-storage capacity of the respective neuronal circuits (Moser et al., 1998). I therefore hypothesized that some fast-acting homeostatic mechanism might be in place that, after a certain threshold of overall potentiation is reached, limits further synaptic strenghening at heterosynaptic sites. To test this hypothesis, I performed long-lasting extracellular and intracellular electrophyiological recordings in acute hippocampal slices of rats using the classical NMDA-receptor dependent CA3-CA1 LTP.

In a first series of experiments, I observed, that one hour after saturating LTP through repeated tetanizations of one Schaffer collateral pathway, hippocampal CA1 neurons still exhibited additional potentiation at heterosynaptic sites in response to a LTP stimulus in a second independent pathway. Neither the amount nor the persistence of this LTP differed from the LTP induced in naïve control slices that had not sustained saturating LTP induction. These findings argued against homeostatic protection against LTP saturation, but were not conclusive, as it is possible that the number of synapses activated by the first stimulus was too low to trigger a homeostatic down-regulation of the remaining synapses' ability to undergo LTP.

In an attempt to overcome such a possible threshold, I performed experiments using chemical potentiation to induce very widespread synaptic strengthening throughout the slice. Initial experiments confirmed that chemical LTP was NDMA-receptor dependent and previous chemical potentiation occluded later tetanic LTP induction, indicating that both forms of potentiation share common intracellular signaling pathways. I then used a local superfusion technique to selectively spare a small number of synapses from being chemically potentiated. Pharmacological blockade of synaptic transmission outside the superfusion spot allowed me to electrophysiologically isolate the inside-spot synapses. I then tested whether these superfused, and hence unpotentiated, synapses inside the spot were still capable of exhibiting LTP. My data show, that under these conditions the inside-spot synapses did not exhibit potentiation. Control experiments established that LTP can be induced at inside-spot synapses if prior chemical potentiation has not occurred. These findings strongly support the presence of a homeostatic mechanism under circumstances in which a sufficient proportion of a neuron's synapses have previously undergone LTP.

2 Introduction

Millions upon millions of individual nerve cells compose the unitary building blocks of the nervous system. Nerve cells make contact and communicate with each other at synapses, highly specialized structures for the chemical transmission of information. A principal neuron bears many thousands of synapses, all of which can be subject to activity-dependent changes, such as weakening or strengthening of their efficacy. This is referred to as synaptic plasticity. The theoretical concept of activity-dependent plasticity was developed in the late nineteenth century and was refined by Donald Hebb in the 1940s (James, 1890; Cajal, 1894; Hebb, 1949). It was experimentally confirmed with the discovery of long-term potentiation (Bliss and Lømo, 1973). Synaptic plasticity is widely regarded as the cellular correlate of learning and memory and hence vital to any organism.

However, Hebbian plasticity promotes changes in neuronal activity in such a powerful way that it presents the danger of driving neurons or neuronal networks beyond their optimal operating range. Thus, to ensure proper functioning, the nervous system – as do many complex systems – has to balance two seemingly conflicting requirements: change versus stability. In fact, these requirements are opposing but complementary and a variety of homeostatic mechanisms have been identified that help to maintain a dynamic equilibrium between the two. Most homeostatic mechanisms have been described for developing neuronal circuits; I set out to investigate whether in the adult nervous system homeostatic intervention is present to counteract the potent effects of Hebbian plasticity.

2.1 Hebbian plasticity

Hebb's famous postulate

"When an axon of cell A… excites cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's efficiency as one of the cells firing B is increased."
(Hebb, 1949)

proposes a coincidence detection rule: synapses between two cells are strengthened when there is correlated pre- and postsynaptic activity. The first synapses with this property were identified in the hippocampus (Lømo, 1966; Bliss and Lømo, 1973; Bliss and Garner-Medwin, 1973). Brief high-frequency stimulation of a neuronal pathway can lead to long-lasting changes in synaptic strength, a phenomenon termed long-term potentiation (LTP). Long-term potentiation is inducible within milliseconds, but it can last - as its name suggests - over long periods of time: many hours in vitro and even weeks in vivo (Bliss and Gardner-Medwin, 1973; Staubli and Lynch, 1987).

LTP has three important characteristics: *input specificity*, *cooperativity* and *associativity* (Fig. 2.1; for reviews see e.g. Bliss and Collingridge, 1993; Malenka, 2003).

Input specificity means that only those synapses become potentiated that were activated by the LTP inducing stimulus. Independent, non-activated synapses on the same neuron do not get potentiated. However, synapse specificity of LTP can break down over short distances (Bonhoeffer, 1989; Schuman and Madison, 1994; Engert and Bonhoeffer, 1997).

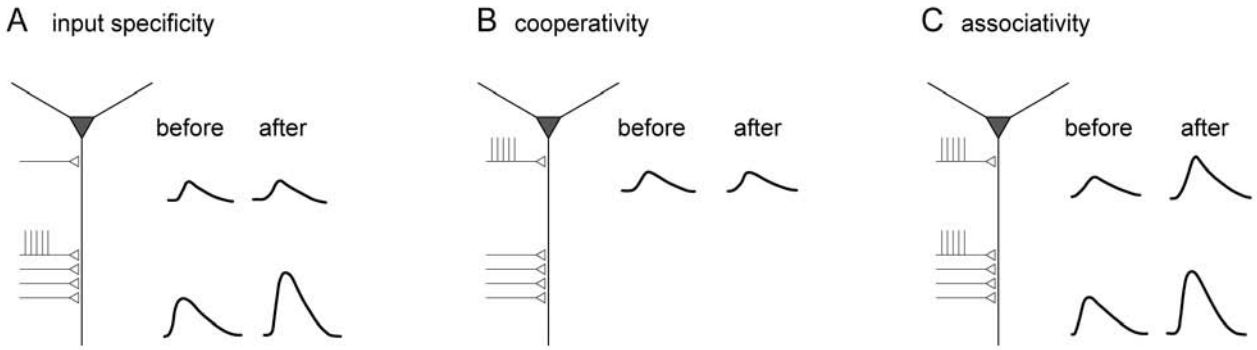


Figure 2.1 Long-term potentiation has three important characteristics: input specificity, cooperativity and associativity. In the figure, a single pyramidal cell receives weak and strong synaptic input from two different groups of axons. (A) Tetanic stimulation of the strong input alone causes LTP in the strong pathway but not in the weak (compare the potential before and after tetanus). (B) Tetanic stimulation of the weak input alone does not cause LTP in this pathway. (C) Tetanic stimulation of the strong and the weak pathways together causes LTP in both pathways. Adapted from Kandel et al., 1995.

Cooperativity refers to the fact that there is an intensity threshold for induction of LTP. When using high-frequency stimulation, a crucial number of presynaptic fibres must be stimulated together, because only their cooperative activity leads to a sufficient postsynaptic depolarization.

Associativity has often been viewed as a cellular analogue of classical conditioning, i.e. a weak, subthreshold stimulus, which would not induce LTP on its own, can do so if paired with a strong stimulus onto the same neuron. The associative property of LTP is well in accordance with Hebb's postulate.

The first direct proof of Hebb's postulate came from Gustafsson and Wigström (1986), who recorded intracellularly from a single hippocampal neuron and used extracellular stimulation to activate presynaptic fibers. They showed that simultaneous neuronal activation by pairing presynaptic fiber stimulation with postsynaptic depolarization persistently increased synaptic strength. However, Bliss and Lømo, who first reported LTP, employed high-frequency stimulation of presynaptic afferents ('tetanus'). So how can presynaptic activation alone induce synaptic potentiation? With a tetanus, many stimulation pulses (normally about 50 to 200 pulses) come shortly one after the other. The first pulse evokes presynaptic action potential firing and a concomitant depolarization of the postsynaptic neuron. The inter-pulse-interval however is so short that the neuron has not yet repolarized to its normal resting potential when subsequent pulses arrive, i.e. the depolarizing events add up. This temporal summation of excitatory postsynaptic potentials (EPSPs) leads to a sustained depolarization of the postsynaptic neuron. Thus, the prerequisite for LTP, namely simultaneous presynaptic activation and neuronal depolarization is met and the synapses become potentiated.

A central feature of Hebb's postulate is temporal specificity: The synaptic connection between two cells is strengthened if cell A 'takes part in firing' cell B, in other words if cell A fires before cell B. Such temporal specificity of synaptic modification may be important for physiological function, especially in the context of associative learning. Recent experiments have revealed the importance of the temporal order of pre-and postsynaptic

action for synaptic modification (Magee and Johnston, 1997; Markram et al., 1997; Bi and Poo, 1998). The same pair of pre- and postsynaptic action potentials can increase or decrease the strength of the synapse. It is the relative timing between the presynaptic and the postsynaptic spiking that determines both direction and extent of synaptic changes. If the presynaptic action potential precedes the postsynaptic one, the synapse becomes potentiated. If it follows the postsynaptic spike, the synapse is depressed. Hence, the so-called spike-timing dependent plasticity (STDP) provides a straightforward learning rule: synapses that contribute to postsynaptic spiking are 'rewarded'; unrelated synapses are punished. The transition between LTP and LTD is very sharp and occurs within a few milliseconds, thus reflecting the sequence of events in a precise fashion. This property of STDP makes it a good cellular correlate for classical conditioning. The conditioned stimulus has to precede the unconditioned stimulus for learning to occur.

An important breakthrough for the mechanistic understanding of LTP induction was the discovery of a special subtype of glutamate receptor, the NMDA receptor (termed after its agonist N-methyl-D-aspartate; Collingridge et al., 1983). The NMDA receptor is a ligand-gated cation channel, which has specific properties that enable it to act as molecular coincidence detector. At normal resting potential of the neuron, the channel-forming pore is blocked by magnesium ions. Two conditions must be met for the NMDA receptor to allow permeation of cations, mainly sodium and calcium. First, the neurotransmitter glutamate released from the presynaptic neuron must bind to the receptor, and second, the magnesium block must be relieved through postsynaptic depolarization. Thus, the NMDA receptor responds to simultaneous activity in the afferent fibres and adequate depolarization in target dendrites, thereby acting as a coincidence detector. Calcium influx, mainly through the NMDA receptor, is crucial for LTP induction and activates different intracellular signaling cascades. Strong evidence denominates calcium-calmodulin-dependent protein kinase II (CaMKII) as a key player in the induction of LTP, but also several other protein kinases have been implicated (Malenka and Nicoll, 1999).

While there is general agreement that induction of LTP takes place on the postsynaptic side, the locus of LTP expression is subject to a longstanding debate (Bliss and Collingridge, 1993). Both pre- and postsynaptic expression mechanisms have been suggested, including increase in the probability of neurotransmitter release and modification in AMPA receptor function or number. AMPA receptors are another subtype of ionotropic glutamate receptors, responsible for baseline synaptic transmission.

Expression and maintenance of LTP can be divided into different time-phases, which may be distinct or partially overlapping. An early phase of LTP, lasting up to one hour, is independent of translation and involves the rapid modulation of existing proteins by phosphorylation or dephosphorylation for instance. Longer lasting forms of LTP in contrast seem to require the synthesis of new proteins (e.g., Frey et al., 1988).

LTP is not unique to the hippocampus, a structure known for its importance in the formation of memories, but is also observed in many other brain structures, including the cerebral cortex and the amygdala. In fact, it seems to be a fundamental property

of the majority of excitatory synapses in the mammalian brain (Malenka, 2003). However, not all forms of LTP are NMDA receptor dependent (e.g. LTP of mossy fibers onto hippocampal CA3 neurons, Johnston et al., 1992), which is called "non-Hebbian LTP"

While LTP causes an increase in synaptic strength, a second form of activity-dependent plasticity can be found in the brain that has the inverse direction of change: Long-term depression (LTD) leads to a reduction of synaptic efficacy and was described (amongst other brain regions) in hippocampus, cortex and cerebellum (Lynch et al., 1977; Mulkey and Malenka, 1992; Dudek and Bear, 1993; for a review see Bear and Malenka, 1994). LTD is regarded as the direct counterpart to LTP and some forms of LTD are also NMDA receptor dependent (Mulkey and Malenka, 1992). Others are NMDA receptor independent and require the activation of metabotropic glutamate receptors (Manabe, 1997). Interestingly, both LTP and LTD can be induced at the same population of synapses; the direction of change being determined by the induction stimulus and the history of the synapse (Bear and Malenka, 1994; Bear, 1995; Abraham and Bear, 1996). Differences in the dynamics and sources of intracellular calcium entry as well as its final concentration might underlie the differential changes in synaptic strength (Malenka et al., 1988; Artola and Singer, 1993).

Activity-dependent modifications of synaptic strength are fundamental to the storage of information in the brain (for review see e.g., Martin et al., 2000). A principal neuron in the hippocampus or neocortex receives thousands of excitatory inputs, all subject to activity-dependent changes in synaptic efficacy, such as LTP or LTD. However, Hebbian (associative) forms of plasticity have particularly strong destabilizing effects on network activity. Essentially, they are positive feedback loops that tend to drive synaptic strengths towards their maximum or minimum values. When some of the inputs onto a postsynaptic neuron become potentiated, the excitatory drive increases and so does the probability that any given input will make the neuron fire an action potential. This positive correlation will strengthen the synapses even further, and the cycle begins again. In an analogous manner, synapses that have been depressed, are on a downward spiral to zero synaptic strength. Without further regulation, LTP and LTD could drive activity levels in neuronal circuits towards runaway excitation or quiescence. Therefore, stabilizing mechanisms are required that preserve the optimal operating range of synapses, neurons and neuronal circuits.

Indeed, in addition to Hebbian plasticity, other forms of plasticity are at work to maintain a dynamic equilibrium in the face of ongoing changes. For example, the ability of neurons to undergo LTP or LTD is not a set property, but is subject to activity-dependent regulation itself (Bienenstock et al., 1982). This 'plasticity of synaptic plasticity' has hence been termed 'metaplasticity' (Abraham and Bear, 1996; Abraham and Tate, 1997). Previous synaptic or cellular activity (or inactivity) can lead to a change in the direction or degree of synaptic plasticity elicited by a given pattern of synaptic activation (Abraham and Bear, 1996). Hence, it is not only a snapshot of concurrent pre- and postsynaptic activity, but also the history of the synapse that is taken into account for synaptic plasticity to occur. Similarly, the regulation of synaptic strength by the prior history of neuronal activation can also be observed in developing neuronal

circuits. When activity levels are altered for prolonged periods of time, neurons can scale their synaptic strength up or down to maintain constant firing rates (e.g. Turrigiano et al., 1998). These observations indicate that neurons can monitor their own activity levels and possess strategies to maintain a preferred level of activity. Hence, they are important examples for neuronal homeostasis.

2.2 Homeostasis in the nervous system

Homeostasis is one of the most basic cellular processes by which a cell responds to a change in the intracellular or extracellular environment to maintain a constant physiology (see InfoBox). As do all cells in a living organism, nerve cells possess homeostatic mechanisms to take care of

InfoBox: Homeostasis

The concept of homeostasis was first introduced by the French Claude Bernard in 1865. In his 'Introduction to Experimental Medicine' he noted that it was essential for the survival of an organism to maintain what he called 'la fixeté du milieu interieur' or the constancy of the interior environment (Bernard, 1865; also see Conti, 2002). The term itself was coined decades later by the American physiologist Walter Cannon from two Greek words 'homeo' and 'stasis' meaning 'to remain the same'. In his book, the 'Wisdom of the Body' (1932) Cannon wrote "The coordinated physiological processes which maintain most of the steady states in the organism are so complex and so peculiar to living beings--involving, as they may, the brain and nerves, the heart, lungs, kidneys and spleen, all working cooperatively--that I have suggested a special designation for these states, homeostasis. The word does not imply something set and immobile, a stagnation. It means a condition--a condition which may vary, but which is relatively constant."

Not only has homeostasis become a central concept for physiology, but it was also adopted by other disciplines, e.g. cybernetics. Homeostasis applies to physiological processes but also to engineered feedback loops such as thermostat control. The technical example of thermostatic heating control, explains the components of a homeostatic control system. A certain characteristic of the environment, e.g. temperature, is the variable controlled by the system. A sensor – in this case a thermometer – detects changes in the variable and feeds that information back to a control centre, the detector.

The detector compares the actual value of the variable to a pre-determined set-point value. If the actual value deviates from the set-point, a signal is sent from the integrator in order to turn on the heating. A mechanism or an instrument that has an effect on the variable is called an effector. Human body thermoregulation functions much in the same way. Nerve receptors monitor body temperature, which is regulated centrally by the hypothalamus. If body temperature drops too low or rises too high, a signal will be sent to the appropriate effector organs: muscle shivering or sweating are induced to bring body temperature back to its set-point of 37°C. Temperature control is only one of many examples for homeostatic regulation of physiological processes. The concept of homeostasis applies to ecosystems, organisms, cells, biochemical signalling pathways, but also to complex social systems.

basic housekeeping function such as oxygen consumption, energy metabolism and regulation of ion concentration. In addition to keeping the cellular machinery running, neurons face an extra task – the regulation of neuronal activity. Maintaining proper functioning is a ubiquitous challenge for all kinds of nervous systems and accordingly, homeostatic mechanisms have been described in a number of organisms, in vertebrates as well as in invertebrates, in the central as well as in the peripheral nervous system, in the developing as well as the mature nervous system (for review see e.g. Turrigiano, 1999; Davis and Bezprozvanny, 2001; Turrigiano and Nelson, 2004). These include mechanisms for adjusting the intrinsic excitability of neurons, mechanisms for adjusting synaptic strength and mechanisms for regulating Hebbian plasticity itself.

2.2.1 Homeostasis of the intrinsic electrical properties of neurons: The example of pattern generation

In the example of rhythmic pattern generation, the set-point of the neuronal system is clearly defined, namely to generate a specific pattern of activity. Neuronal pattern generation is often 'implemented' by a network of neurons that are rhythmically active. Therefore, it is important, that the intrinsic electrical properties of the neurons that are part of a pattern generator are regulated precisely. Homeostatic regulation of intrinsic excitability has been observed in an invertebrate model system of a neuronal pattern generator: the lobster stomatogastric ganglion (reviewed in Turrigiano, 1999).

The lobster stomatogastric ganglion contains a network of rhythmically active neurons. The bursting properties of these neurons are driven by a balance between intrinsic ionic conductances and patterned excitatory, inhibitory and modulatory synaptic input. When acutely isolated from their normal circuitry, neurons of the stomatogastric ganglion show a remarkable capacity to regenerate their bursting properties. Examination of single cells in isolated cell culture demonstrates that single cells can regenerate their endogeneous rhythmic bursting properties by modifying the balance of their ionic conductances (Turrigiano et al., 1994; Thoby-Brisson and Simmers, 1998; Golowasch et al., 1999). Thus, compensatory mechanisms are in place that reinstall rhythmic activity. A similar phenomenon has been observed in the vertebrate spinal chord (Chub and Donovan, 1998; Galante et al., 2001).

The example of pattern generation shows that excitability of neurons can be regulated by homeostatic mechanisms. Besides regulation of intrinsic electrical properties, also synaptic efficacy can be target of homeostatic regulation.

2.2.2 Synaptic homeostasis in the peripheral nervous system: The example of the nerve-muscle-synapse

The nerve-muscle synapse is one of the best-defined model systems to analyse the regulation of synaptic efficacy, because a single muscle fiber is usually innervated by only one motor neuron. In contrast, a postsynaptic neuron in the central nervous system can

have many thousands of presynaptic partners, which complicates matters tremendously. Therefore, basic properties of synaptic transmission have been studied at the neuromuscular junction (NMJ) extensively and it has also become a classic model system for synaptic homeostasis.

At the NMJ, the maintenance of synaptic efficacy is complicated by the large increase in muscle volume that takes place during development, which requires a concomitant increase in synaptic strength to assure appropriate muscle function. Accordingly, the changes in the synaptic properties of the pre- and postsynaptic partners must be precisely coordinated during the growth and development of the NMJ, so that the muscle remains functional at all times.

Homeostatic compensatory mechanisms are particularly well documented for the *Drosophila melanogaster* NMJ (for review see Davis and Bezprozvanny, 2001): Rapid growth of the larvae and genetic tools make this a particularly well-suited model organism. The *Drosophila* NMJ is glutamatergic. Independent genetic manipulations have been used that alter postsynaptic glutamate receptor function (Petersen et al., 1997; Davis et al., 1998) or change the innervation pattern leading to increased or decreased muscle innervation (Davis et al., 1997). Physiological experiments at these genetically manipulated synapses have demonstrated that distinct, target-specific homeostatic mechanisms regulate synaptic efficacy to compensate for altered innervation or altered postsynaptic excitation. Presynaptic release or quantal size is regulated appropriately to ensure normal muscle depolarization. Thus, at the NMJ, homeostatic compensation can take place both pre- and postsynaptically.

2.2.3 Synaptic homeostasis in the central nervous system: The example of synaptic scaling

Both lobster stomatogastric ganglion and the neuromuscular junction are structures that are expected to have a reliable and reproducible mode of activity – for example rhythmic pattern generation or consistent activation of muscle fibres. Maintaining stability here seems to be a relatively straightforward task, as the desired set-point of the homeostatic system is obvious. For a neuron in the cortex or hippocampus, the situation is different and more delicate. Such a principal neuron has to integrate inputs from hundreds or thousands of synaptic partners; which can be of excitatory, inhibitory or modulatory nature. Moreover, all of these synapses can undergo plastic changes. As a consequence, activity in these neurons can vary drastically, and over short timescales, these fluctuations have to be allowed, because they carry information. Over longer timescales, however, stability constraints apply: extremes such as overexcitation or complete silence have to be avoided for the neurons to remain functional in a circuit. Instead of a set-point, it seems more appropriate to think of an optimal range of neuronal activity where overshoots or undershoots of firing rates have to be prevented.

Indeed, there is good evidence for homeostatic control of firing rates in central neurons. Mechanistic studies of this type of homeostasis usually rely on pushing activity levels to extremes – either a complete block or very high activity levels. Central neurons in culture form excitatory and inhibitory networks that develop spontaneous activity. Prolonged pharmacological blockade of spiking activity or glutamatergic synaptic transmission in these cultures generates a rebound

phenomenon: during drug application, activity is blocked, but after removal of the drug, the network exhibits increased excitability and is hyperactive (Ramakers et al., 1990; Corner and Ramakers, 1992). The reciprocal manipulation – increasing network activity by partial removal of inhibition – leads to an initial increase in firing rate, but over a two-day period, firing rates fall until they resemble control levels (Fig. 2.2A; Turrigiano et al., 1998).

Turrigiano and colleagues sought to determine the mechanistics underlying this adjustment of firing rates. They used primary cell cultures of visual cortical pyramidal neurons and globally blocked or increased activity by 48 hour application of drugs: TTX to abolish spiking, CNQX to block AMPA receptor functioning, which indirectly prevents spiking, and bicuculline, blocking $GABA_A$-mediated inhibition, thereby increasing firing rates. Subsequently, they recorded miniature excitatory postsynaptic currents (mEPSCs) from voltage-clamped neurons. Miniature EPSCs arise from random spontaneous transmitter release of presynaptic vesicles at many different synaptic sites. Thus, mEPSC recordings allow measuring the strength of a large number of a neuron's synapses. Turrigiano and colleagues observed that activity-blocked neurons had larger mEPSC amplitudes than control neurons whereas the mEPSC amplitudes in activity-enhanced neurons were smaller than in control neurons. Thus, these neurons regulated their own firing rates by scaling the strength of their synaptic inputs up or down as a function of activity. Hence, this phenomenon was termed 'synaptic scaling'. Cumulative plots of mEPSC amplitudes revealed that the entire activity-blocked distribution is shifted towards larger values, while the activity-enhanced distribution is shifted towards smaller amplitudes. Both distributions can be transformed into the control distribution almost perfectly by multiplying (or dividing) each amplitude value by a constant, suggesting that synaptic scaling is multiplicative.

Multiplicative synaptic scaling occurs through increases or decreases in the quantal amplitude of AMPA-mediated synaptic transmission, seemingly at all of the synapses on a neuron and probably as a result of changes in postsynaptic AMPA receptor number (Turrigiano et al., 1998). Extreme changes in activity also appear to regulate NMDA receptor expression under some conditions (Rao and Craig, 1997; Watt et al, 2000).

There is evidence that synaptic scaling in these cultured cortical networks is mediated through activity-dependent release of the neurotrophin brain-derived neurotrophic factor (BDNF; Rutherford et al., 1998). Interestingly, BDNF has opposite effects on the excitatory synapses onto (excitatory) pyramidal neurons and (inhibitory) interneurons, thereby coordinating changes in excitation and inhibition. Furthermore, activity deprivation reduces miniature inhibitory postsynaptic current amplitude by decreasing the number of postsynaptic $GABA_A$ receptors (Kilman, 2002). Synaptic scaling was also shown to operate in vivo in the developing visual cortex following manipulations of visual experience (Desai et al., 2002).

Synaptic scaling differs from Hebbian synaptic plasticity, such as LTP or LTD, in three important points. First, it is independent of NMDA receptor activation (Turrigiano et al., 1998). Second, it is relatively slow, requiring hours or days of altered activity to modify synaptic strengths. Third, it is a global

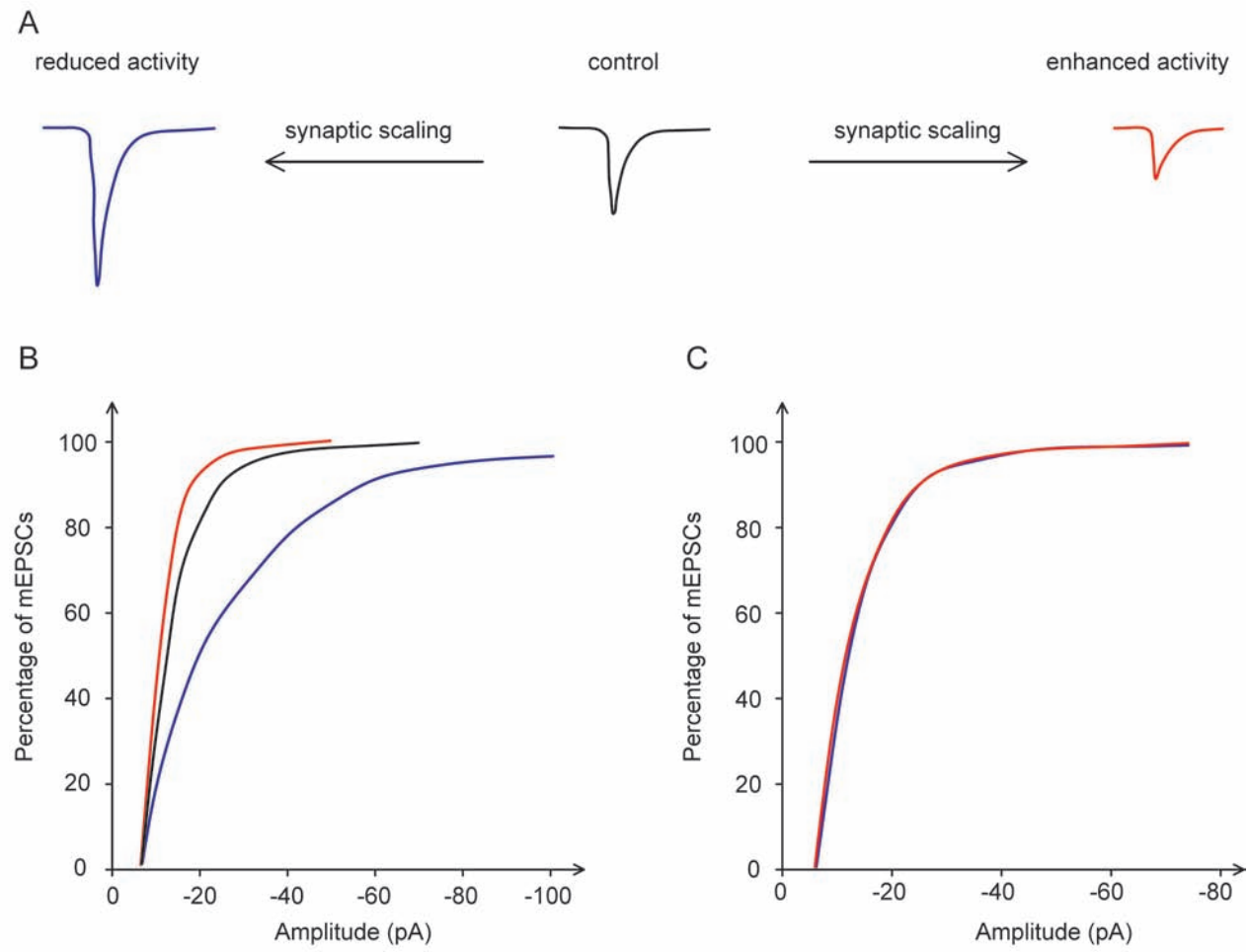


Figure 2.2 Synaptic scaling. (A) Increased activity reduces the amplitude of miniature excitatory postsynaptic currents (mEPSCs) onto cortical pyramidal neurons. Decreased activity has the opposite effect, indicating that quantal amplitude is regulated in a homeostatic manner by prolonged changes in activity. (B) Plotting mEPSC amplitudes as a cumulative histogram shows that the entire distribution of amplitudes is increased (reduced activity, blue curve) or decreased (increased activity, red curve). (C) If these distributions are scaled up or down by multiplying each value in the experimental distribution by the same factor, they overlay the control distribution almost perfectly, indicating that all excitatory synapses onto pyramidal neurons are scaled up or down multiplicatively. Adapted from Turrigiano and Nelson, 2004.

phenomenon, scaling all synapses of a neuron up or down with the same multiplicative factor (Fig. 2.2B and C; Turrigiano, 1999).

Global manipulations of neuronal activity that lead to synaptic scaling in cortical neurons may have different effects in other brain structures. In cortical cultures, chronic silencing of all neurons results in larger quantal amplitude with little effect on the number of synapses or mEPSC frequency, indicating a postsynaptic mechanism (see above). Similar manipulations in hippocampal neurons in culture cause only a modest increase in the quantal amplitude, but a large increase in the frequency of mEPSCs (Bacci et al., 2001; Burrone et al., 2002; Thiagarajan, 2002; for a critical discussion see Burrone and Murthy, 2003; Turrigiano and Nelson, 2004). Increases in mEPSC frequency are traditionally interpreted as a presynaptic change; and indeed, increase in the probability of presynaptic transmitter release and changes

in presynaptic structure and function have been demonstrated in inactive hippocampal neurons (Murthy et al, 2001). Another postsynaptic explanation could be that in hippocampal neurons, silent synapses get unveiled after AMPA receptor insertion at postsynaptic sites (Liao et al., 1999). As more synapses become functional, mEPSC frequency increases.

Synaptic scaling is not only observed as a response to altered firing rates, but also as compensatory mechanism for electrotonic distance. This form of synaptic scaling observed in hippocampal CA1 neurons sets synaptic strength as a function of the distance of a synapse to the soma and was hence called 'distance-dependent scaling' (Smith et al., 2003). This might compensate for reduced synaptic efficacy of synapses further apart from the spike-initiation zone at the axon hillock, because of increased electrotonic distance. In neocortical neurons, distance-dependent scaling was not observed (Williams and Stuart, 2002).

2.2.4 Homeostatic regulation of Hebbian plasticity: The example of metaplasticity

Metaplasticity in the most general sense denotes the phenomenon that plasticity rules themselves can be plastic. Thus, the very same stimulation pattern can yield different results, depending on the history of the synapse. Consequently, the induction of synaptic plasticity is not only determined by the momentary Hebbian conditions, but also by integrating neuronal activity over longer periods of time.

It is commonly observed that repeated tetanization episodes eventually lead to a state in which no further LTP can be elicited ('saturation' of LTP, e.g. Frey et al., 1995). In this instance, the very same tetanus that elicits LTP when the synapses are still naïve, is no longer effective once the synapses are 'pre-potentiated'. Interestingly, metaplasticity can also be elicited by 'priming stimulation', which in itself does not cause lasting changes in synaptic strength. Huang and colleagues (1992) have demonstrated that a strong tetanus failed to induce LTP in hippocampal CA1 neurons if weak tetani were previously delivered to the same Schaffer collateral input pathway. Although the weak tetani caused only transient increase, they interfered with LTP induction later on. The inhibition of LTP in these experiments was not absolute, however, because it could be overcome by stronger tetanic stimulation (Huang et al., 1992). Thus, in this case, prior stimulation did not block plasticity per se, but raised the stimulation threshold for LTP. By making further LTP induction harder and harder, the positive feedback loop of Hebbian plasticity rules is broken.

Furthermore, it also has been observed that the same stimulation patterns that inhibit subsequent LTP induction facilitate the induction of LTD (e.g., Barrionuevo et al., 1980; O'Dell and Kandel, 1994; Larkman et al., 1992). This facilitated LTD has also been called 'depotentiation' (e.g. Fuji et al., 1991; Bashir and Collingridge, 1994). All these instances of metaplasticity are homosynaptic, i.e. regulation of LTP induction is observed at the same synapses that had been subject to prior activation.

In summary, induction of LTP at a certain subset of synapses makes further LTP on these synapses more difficult and facilitates LTD. Hence, metaplasticity is a form of homeostasis that acts to maintain the equilibrium and to balance out the effect of Hebbian plasticity.

2.3 Scope of this study

The precise regulation of neuronal activity is essential for proper nerve cell, neural circuit, information storage and nervous system function. During development and throughout life, neurons are challenged with perturbations that can alter their activity levels, including changes in cell size, innervation patterns and strength of synaptic input. There is increasing experimental evidence demonstrating that neurons are able to compensate for these types of perturbations and maintain their function within normal physiological range. These experiments suggest that neurons have the capacity to monitor their own activity levels and transduce this information into regulated changes in excitability or synaptic strength. The ability to learn and to adapt to our environment is thought to require changes in synaptic connectivity and neural excitation. It is essential, therefore, that homeostatic regulation of neuronal activity does not preclude activity-dependent modification of neural circuitry. Rather, homeostatic mechanisms might establish limits beyond which activity-dependent changes may not reasonably modify cellular activity. Thus whereas synaptic competition and activity-dependent changes such as long-term potentiation or long-term depression encompass mechanisms that can generate precise change in synaptic structure and function, homeostasis is thought to constrain this change within reasonable physiological limits.

A pyramidal neuron in the hippocampus receives many thousands of excitatory inputs, all subject to activity-dependent changes in synaptic strength. If too many synapses on a neuron get potentiated, the neuron might be at risk of overexcitation and excitotoxic damage. In addition, selective differences in synaptic weight would diminish, thereby reducing the information-storage capacity of neuronal circuits. In other words, the operating range of the single neuron as well as the neural network might be seriously compromised. It has been shown that both developing and mature nerve cells possess homeostatic mechanisms to adjust their operating range. One of these mechanisms impairs further LTP induction on synapses that had been potentiated before. I posed the question whether neurons also possess a homeostatic mechanism that regulates LTP induction at hitherto non-potentiated synapses. Such a mechanism would come into play once a certain proportion of synapses have been potentiated and it would limit further synaptic strengthening at other synapses on the same neuron. To test this possibility, I performed electrophysiological experiments in rat hippocampal slices to study the classical NMDA receptor dependent Hebbian CA3-CA1 LTP.

3 Methods

3.1 The hippocampus

The hippocampus is a standard model for the investigation of LTP. The hippocampal formation plays an important role in learning and memory (e.g. Squire and Zola-Morgan, 1988) and was the first brain structure where LTP was experimentally observed (Bliss and Lømo, 1973). The most prominent cell type in the hippocampus is the pyramidal neuron; additionally, there are a number of different inhibitory interneurons. Morphological differences of the pyramidal neurons allow one to distinguish the hippocampal area CA3 from area CA1 (see Fig. 3.1A and C). The somata of the pyramidal neurons are located in the pyramidal cell layer (*stratum*

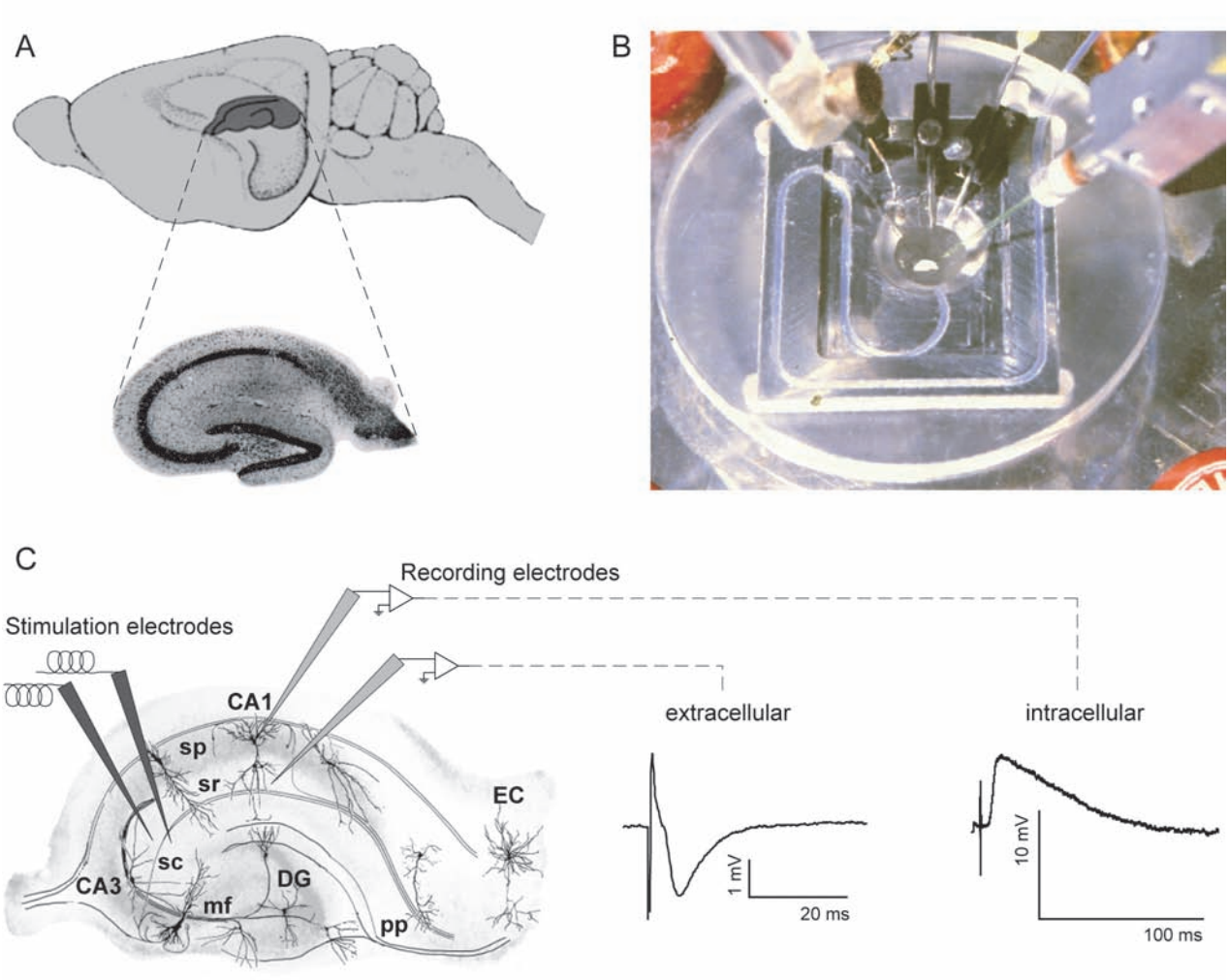


Figure 3.1 The hippocampus. (A) Orientation of the left hippocampus in the rodent brain. The hippocampus is taken out and cut into transverse slices. Photograph: Nissl staining of a hippocampal slice. (B) Hippocampal slice in a submersion recording chamber. A stimulation electrode is coming from the left, the recording electrode is seen on the right. (C) Electrophysiology in hippocampal slices. The stimulation electrodes were positioned in the Schaffer collateral axons in area CA3. For extracellular recordings of fEPSPs, a recording electrode was inserted in stratum radiatum of area CA1. For intracellular recordings, a single CA1 neuron was penetrated with a sharp recording electrode. CA: cornus ammoni, DG: dentate gyrus, EC: entorhinal cortex, sc: Schaffer collaterals, sp: stratum pyramidale, mf: mossy fibers, pp: perforant pathway. Modified after Korte, 2001.

pyramidale). A CA1 neuron extends its apical dendrites into the *stratum radiatum* and its basal dendrites into the *stratum oriens*. The hippocampus contains a trisynaptic excitatory pathway: First, the perforant pathway, which connects the entorhinal cortex to the dentate gyrus. Second, the mossy fiber pathway leading from the dentate gyrus to area CA3 and third, the Schaffer collateral pathway, connecting pyramidal cells from area CA3 to pyramidal cells in area CA1. The hippocampus is organized in lamellae, extending along the longitudinal axis. Thus, when the hippocampus is cut in transverse slices, the connectivity between neurons is retained, as each slice contains a complete trisynaptic pathway. This stereotyped cytoarchitecture makes the hippocampus well suited for in vitro studies.

3.2 Preparation of acute hippocampal slices

Transverse hippocampal slices were prepared from male Wistar outbred rats (4-6 weeks old, in-house colony, animal house MPI of Neurobiology/MPI of Biochemistry). Rats were briefly anesthetized with halothane and decapitated with a custom-built laboratory guillotine. The skull was opened and the whole brain was rapidly removed and placed into ice-cold artificial cerebrospinal fluid (ACSF). ACSF for preparation and electrophysiological recordings was the same, but the composition was slightly different for each of the experimental series (see Table 3.1 and below). Fast dissection and immediate cooling is necessary to prevent oxidative stress due to increased neuronal activity caused by the preparation procedure. The ACSF was continuously aerated with carbogen (95% O_2, 5% CO_2) to maintain a stable pH (7.4) and to saturate the solution with oxygen. After cooling the brain for two minutes, the hemispheres were separated and the hippocampus was carefully dissected from

Components (in mM)	ACSF 1 extracellular	ACSF 2 intracellular	ACSF 3 superfusion	Blocking medium	Potentiation medium	Superfusion medium
NaCl	124	120	120	120	124	120
KCl	3	3	3	3	5	3
NaH_2PO_4		1.2	1.2	1.2	1.25	1.2
KH_2PO	1.25					
$MgSO_4$	2					
$MgCl_2$		1.2	1.2	3.6	0.1	0.9
NaHCO	26	23	23	23	24	23
$CaCl_2$	2.5	2.4	2.4	1.2	5	10
d-Glucose	10	11	11	11	10	11
$CdCl_2$				5µM		
TEA					25	
Phenol red						0.5

Table 3.1 Composition of different ACSFs.

one hemisphere while the other hemisphere remained cooled. The first hippocampus was transferred back into ice-cold ACSF and the second hippocampus was taken out subsequently, i.e. both hippocampi were dissected before further preparation steps were performed. Direct immersion of the first hippocampus facilitates its oxygen and glucose supply while the other hippocampus is being processed. Hippocampi were cut into transverse slices using a so-called egg-slicer (Katz, 1987) or a vibratome (Leica VT 1000S, Germany) according to the requirements of the different experimental series. The egg-slicer separates 400µm thick hippocampal sections by a fine gold-coated tungsten wire (California Fine Wire, USA) net that falls down onto the slice in a guillotine-like fashion. The sections were then separated with fine forceps and transferred into a storage chamber with a pipette. Subsequently, the second hippocampus was prepared in the same way. Egg-slicing is a very fast and efficient method of tissue sectioning with a total time of only seven to eight minutes from brain preparation to slice storage. The rapid time course ensures minimal cellular damage due to oxygen deprivation. However, the fast, crushing way of cutting also ruptures a substantial amount of cells on the slice surface. Therefore, this procedure was used for extracellular and intracellular experiments where the recording depth in the slice was between 100µm and 200µm.

When recordings had to be performed on the surface of the slice, as in the superfusion experiments, the more gentle technique of vibratome sectioning was used. A small agar block (1.6% agar dissolved in 0.9% NaCl) was glued onto the specimen holder with cyanoacrylate adhesive (Sekundenkleber, UHU, Germany) and a thin film of the same adhesive was spread out onto the holder to fix the tissue. The hippocampus was trimmed from both sides and was then placed in front of the agar block in an upright position. Due to the trimming procedure, the yield of slices is lower and therefore sections were cut with a thickness of 350µm. An ethanol wiped razor blade (Fine Science Tools, Germany) was used for cutting and the hippocampus was continuously bathed in ice-cold, carbogenated ACSF during the procedure. Single sections were picked up with a pipette and placed into a storage chamber.

ACSF in the storage chamber was at room temperature and constantly bubbled with carbogen. In the storage chamber, both vibratome and egg-slicer sections were kept submerged, i.e. each slice was completely surrounded by medium. Slices were allowed to rest for at least one hour before an experiment was started so that neuronal activity could recover to baseline.

3.3 Electrophysiology

ACSF for preparation and electrophysiological recordings was the same and was freshly prepared every day from powdered chemicals (Merck, Germany, see Table 3.1). A different ACSF was used for extracellular, intracellular and superfusion experiments respectively (ACSF1, ACSF2 and ACSF3; see Table 3.1). The ACSF for the intracellular recordings contained less potassium than that for the extracellular recordings. Potassium was slightly reduced to avoid having the resting membrane potential of the CA1 neurons approach the spiking threshold. For the superfusion experiments, it was taken care that the ACSF, and the blocking, potentiation

and superfusion media had approximately the same pH and osmolarity. The ACSF used for the chemical LTP pilot experiments was the same as for the superfusion experiments.

Recordings were performed at a temperature of 32±0.2°C in a submersion recording chamber (Fig. 3.1B). The steel body of the recording chamber could be heated through a special heating wire (Thermocoax 1NcAc 10, Thermocoax, Germany) glued into a groove on the bottom of the chamber with special heat-conducting glue (Bürklin, München, Germany). A temperature probe in the chamber was coupled to a controlling device (DB-1000, Mawitherm, Germany), which regulated the heating current appropriately to maintain the temperature constant at 32°C (±0.2°C). The chamber was continuously perfused with carbogenated ACSF driven by a Minipuls pump (Abimed, Germany) at a rate of 1ml/min. The last part of the ACSF delivery tube went through the heated steel body, whereby the ACSF was warmed before finally reaching the chamber. The efflux cannula was polished obliquely and positioned in an angle of 45° to suck both medium and air to maintain the medium level constant. For stable recordings it is crucial to keep pumping effects (pulsing, changes in medium level) to a minimum. During intracellular recordings, the position of the slice was fixed with a platinum ring. Initially, a net of fine nylon wires was used, but the wires cut into the surface of the slice so that the slice could not be repositioned. A better option was single hairs, attached to only one side of the ring, which had the right weight and flexibility to hold the slice without visible damage.

3.3.1 Stimulation

Monopolar tungsten electrodes were used for stimulation. Tungsten wire (thickness 130µm, TW8-3, Science Products, Germany) was electrolytically sharpened through repeated dipping into an edging solution consisting of 71g $NaNO_2$ and 34g KOH in 100ml H_2O. Subsequently, electrodes were coated with resin (Epoxylite Resin, EPR-4, Clark, England) to isolate them electrically, only the very tip was spared to allow conductance. For the intracellular and the superfusion experiments, more durable prefabricated stimulus electrodes were used (AMS5753, AM Systems, obtained through Biotrend, Germany).

Stimulus isolators (A360, WPI, Germany), driven by a Master8 pulse generator (A.M.P.I., Israel), delivered square stimulation pulses of 0.1 or 0.2ms length and a stimulation strength between 20 - 150µA. An indifferent electrode (Ag-AgCl pellet, E201, Science Products, Germany) was present in the bath solution. Stimulation electrodes were mounted on mechanical (Leitz, Germany) or computer-programmable micromanipulators (Luigs & Neumann, Germany). Visual control through a stereomicroscope facilitated the fine positioning of the electrodes in the CA3 region. In most experiments, two different fascicles of the Schaffer collateral pathway were stimulated (Fig 3.1B)..

In these experiments, the independence of the two stimulation pathways was tested by cross paired-pulse facilitation (PPF). PPF denotes the observation that when the same population of synapses is activated two times within a short time interval (20-50ms), the second excitatory postsynaptic potentials (EPSP) is larger than the first, although the

stimuli are the same. This is thought to be a presynaptic phenomenon; residual calcium in the presynaptic bouton facilitates transmitter release upon the second stimulation pulse, which results in a larger postsynaptic potential. In my experiments, to verify the independence of two stimulation pathways, each pathway received a single test pulse; the time interval between the two pulses was 30ms. If the two pathways were completely independent, i.e. if each stimulation electrode activated distinct and non-overlapping group of Schaffer collateral axons, no PPF would be observed. However, if the two pathways were not independent, PPF would be observed because some synapses were activated twice. I performed the cross PPF assay in both directions (pathway One followed by pathway Two and the other way round), and could therefore exclude an overlap of the two pathways very reliably.

In some of the one-pathway experiments, a second pathway was used to monitor the stability of the electrophysiological recording. The frequency of baseline test stimulation was 0.05Hz or 0.03Hz and the two pathways were stimulated in an alternating fashion.

3.3.2 Extracellular recording

Field excitatory postsynaptic potentials (fEPSPs) were recorded in the stratum radiatum of the CA1 region using glass microelectrodes filled with 3M NaCl (tip resistance 5-20MΩ). Electrodes were pulled from borosilicate glass capillaries with an omega filament (1mm outer diameter, Harvard Apparatus, UK) using a horizontal electrode puller (Flaming Brown P97, Sutter, USA). Filled electrodes were inserted into an electrode holder with an Ag-AgCl pellet (WPI, Germany) and mounted on a computer programmable micromanipulator (Luigs & Neumann, Germany).

Recording electrodes were positioned under visual and auditory control. The voltage at the tip of the recording electrode was made audible by a voltage-frequency converter (MPI of Biological Cybernetics, Germany) together with an audio-monitor. Thereby, slight changes in voltage could easily be detected acoustically.

For the extracellular experiments, stimulation strength was set to elicit 50% of the maximal signal amplitude. For the intracellular experiments, stimulation strength was set to elicit a clear EPSP, which remained below the action potential threshold. In the superfusion experiments, most of the time the maximal stimulation strength of 150μA was used to evoke large-enough fEPSPs even in the blocking phases of an experiment. If the amplitude of the fEPSP was smaller than 0.2mV during the first blocking period, the experiment was stopped. In the second superfusion series, I used the first blocking period to readjust the position of the stimulation electrode, the superfusion spot and/or the recording electrode if the fEPSP of the inside-spot synapses was too small. The proper positioning of these components is crucial to obtain decent signal amplitudes, and small changes in the configuration can have remarkable effects on the size of the fEPSP.

3.3.3 Intracellular recording

Sharp glass electrodes were used to prevent a rundown of cells due to wash-out of cytosolic substances (same glass and electrode puller as

for extracellular recordings). Electrodes were filled with 3M KCl and had a tip resistance of 70-100MΩ. Visual control facilitated the positioning of the electrode above the cell layer of the CA1 region. Subsequently, the electrode was lowered into the slice, and its resistance and capacitance was compensated. The electrode was 'blindly' moved downwards in micrometer steps. A change in the auditory signal indicated when the electrode was in contact with a neuron whereupon a short 'buzz', i.e. a brief oscillation of the electrode, facilitated penetration of the cell membrane. Initially, neurons were hyperpolarized to about –80mV to stabilize the recording. Once the neuron recovered to normal resting potential (usually within 5-15 minutes), the hyperpolarizing current was slowly relieved.

In general, only cells that had a resting potential of less than –60mV without holding current were taken into experiment. Stimulation strength was set to elicit clear subthreshold EPSPs during baseline. Sometimes, when the normal resting potential was close to spiking-threshold, the neuron was held slightly hyperpolarized (-70mV to -75mV) with injected DC current. The resting potential was kept constant throughout an experiment and the recording was stopped if the holding current necessary to stabilize the initial resting potential exceeded -0.25nA. Considerable changes of the resting membrane potential not only alter synaptic drive, but they also might indicate beginning cell death. For both reasons, such neurons were excluded from further analysis.

3.3.4 Electrical and chemical LTP

LTP was induced either electrically or chemically. Conventional electrical LTP was induced with high-frequency stimulation of the same stimulation strength as used during baseline conditions. A single 'tetanus' consisted of a train of 100 pulses at a frequency of 100 Hz. For the extracellular experiments, saturation of LTP was defined operationally by the following three rules:

1. Apply at least three episodes of tetanic stimulation with an inter-tetanus interval of 10 minutes.

2. Continue with tetanic stimulation in ten-minute intervals until the increase in fEPSP amplitude from the $(n-1)^{th}$ tetanus to the n^{th} tetanus is less than ten percentage points.

3. Stop the experiment if the potentiation has fallen below 130% one hour after the last tetanus.

For the intracellular experiments, three tetani with a ten-minute inter-tetanus interval were applied to the saturated pathway to save experimental time. In all experimental series, the test and the control synapses were probed for LTP with a single tetanus (100 pulses, 100Hz).

Chemical LTP was induced by a ten minute bath application of potentiation medium which contained increased potassium, reduced magnesium and the potassium channel blocker tetraethylammonium chloride (TEA; Sigma-Aldrich, Germany; see Table 3.1 and Hosokawa et al., 1995).

Pilot experiments assessed the pharmacological sensitivity of chemical LTP using the NMDA-receptor antagonist D-AP5 and the L-type calcium channel blocker verapamil (both from Tocris; obtained through Biotrend, Germany). Stock solutions of the drugs were prepared using 0.1N NaOH as solvent for AP5 and dH_2O for verapamil. Stock solutions were stored frozen and not used longer than eight weeks. For each experimental day, a fresh aliquot of stock solution was diluted to obtain the final concentration desired. The drugs were added to both the normal ACSF and the potentiation medium, the slice was incubated for 20 minutes, before the potentiation medium was applied. The pharmacological sensitivity of chemical LTP was tested using either 50µM AP5 alone or a combination of 100µM AP5 and 30µM verapamil. In the experiments with AP5 alone, the recording electrode was positioned normally, i.e. 100-200µm below the surface of the slice. For the experiments combining AP5 and verapamil, the recording situation should be similar to the superfusion experiments; therefore, the recording electrode was positioned on the surface of the slice, not deeper than 15µm.

Occlusion experiments were performed to test whether electrical and chemical LTP share common postsynaptic mechanisms. Potentiation medium was bath applied and chemical LTP was monitored for one hour. Subsequently, a tetanus was applied to the same afferents. For some experiments, the stimulation strength was reduced, so that the fEPSPs during the second baseline preceding the tetanus had the same amplitude as during the first baseline. In other experiments, the stimulation strength was not reduced, i.e. the fEPSPs during second baseline were larger than during the first.

3.4 Local Superfusion Technique

The local superfusion technique used here was first introduced by Veselovsky and colleagues and has been successfully applied to different experimental questions since (Veselovsky et al., 1996; Engert and Bonhoeffer, 1997; 1999). Local superfusion creates and maintains a spatially defined microenvironment different from the bath solution.

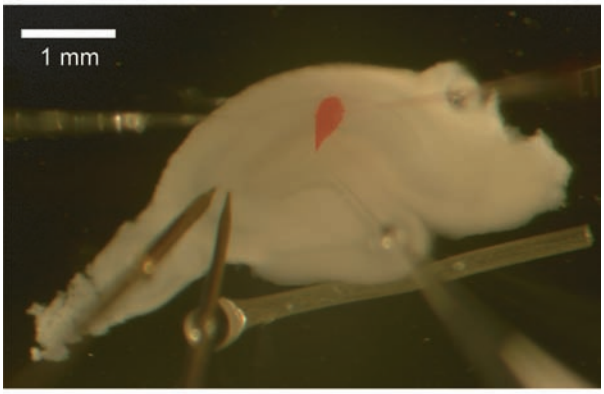


Figure 3.2 Photograph a hippocampal slice and a representative superfusion spot. For better visibility, the colour of the superfusion spot was artificially enhanced. The recording electrode can be seen on the left and the two superfusion pipettes on the right side. Two stimulating electrodes come from bottom left. In some experiments, a second stimulating electrode was used to monitor the stability of the recording.

In the present study, this feature was employed to address the question of homeostatic regulation of synaptic strength. Chemical potentiation was induced throughout the whole slice except for those synapses within the superfusion spot. Afterwards, it was tested whether the locally superfused, hence unpotentiated synapses, had been subject to homeostatic regulation. For this purpose, synapses outside the superfusion spot were silenced pharmacologically by exchanging the normal ACSF with blocking medium, i.e. ACSF containing less calcium (1.2mM instead of 2.4mM), more magnesium (3.6mM

instead of 1.2mM) and a low concentration of cadmium (5µM, Table 3.1).

Cadmium blocks voltage-gated calcium channels, thereby preventing the influx of extracellular calcium into presynaptic terminals and thus the release of neurotransmitters. The combination of reduced calcium concentration and 5µM cadmium in the blocking medium was sufficient to interrupt all synaptic transmission in the remaining slice (Fig. 3.3).

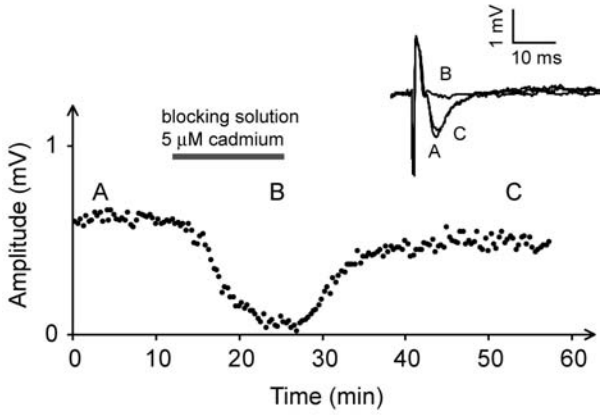


Figure 3.3 Cadmium blocks synaptic transmission in the slice. During the time indicated in the graph, cadmium (5µM) was bath applied: the fEPSP disappeared and recovered after wash-out of cadmium. Representative fEPSPs were taken at the time points indicated in the graph (single sweeps).

3.4.1 Superfusion device

The superfusion device consisted of two glass pipettes placed opposite to each other (Fig. 3.4, 3.5, 3.6). One pipette served for solution delivery and could be connected to different reservoirs located at a slightly elevated position to provide gravitational pressure to the outflowing solution. The second glass pipette was connected to a peristaltic pump that removed the superfusion solution from the external medium (Fig. 3.5).

Both pipettes were pulled from Brand single-use micropipettes (Blaubrand No. 7087 57, Germany) with a BB-CH electrode puller (Mecanex, Switzerland). Puller settings were slightly different so that the delivery pipette had a more elongated and pointed conus whereas the suction pipette was rather short and blunt. The foremost part of the pipette tip was removed using a custom-built microforge to obtain suitable tip openings. The delivery pipette had a tip opening of 30-35µm while the suction pipette opening was 70-85µm, assuring the complete removal of superfusion medium. It was also important that the suction pipette was positioned slightly higher to avoid sucking up the slice. The shape of the pipettes, their absolute tip opening and the ratio between the opening of delivery and suction pipette were critical determinants of size and shape of the superfusion spot.

Both the delivery and the suction pipette were attached to a custom-made manipulator to adjust them toward each other before an experiment. This manipulator itself was then mounted on a computer-programmable manipulator (Luigs&Neumann, Germany), which was used to move the whole arrangement in three dimensions during the

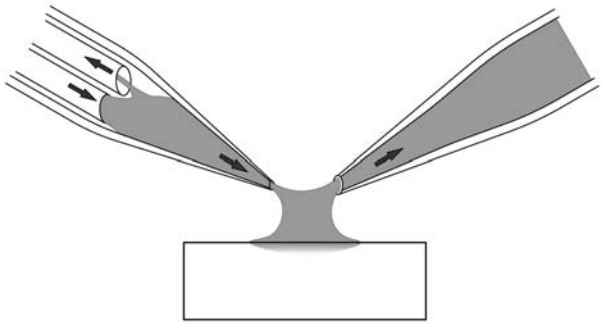


Figure 3.4 Schematic representation of the superfusion device. Left: delivery pipette, right: suction pipette. The superfusion solution penetrates approximately 15-30µm into the slice. An additional suction needle in the delivery pipette enables a quick replacement of superfusion solutions by evacuating the dead space.

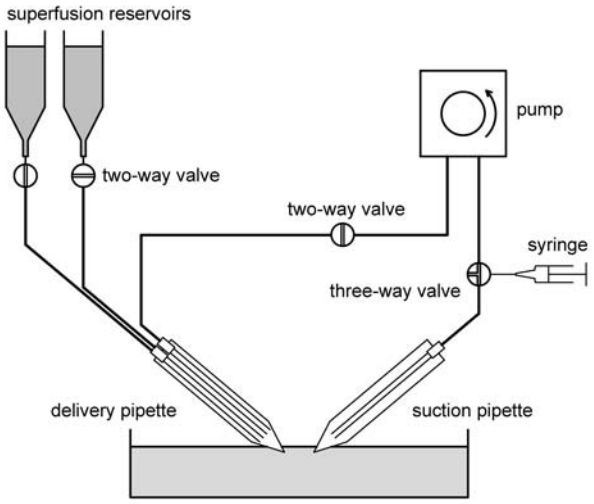


Figure 3.5 Scheme of the multi-barrelled superfusion setup. The delivery pipette can be connected to different reservoirs, the suction pipette is connected to peristaltic pump that removes the superfusion solution from the external medium. A syringe can be used to free the suction pipette in case it is clogged.

recording. The precision in (re-) positioning the spot was estimated to be <1µm. Contact of the superfusion medium to the slice surface was observed as surface tension spreading of the superfusion spot.

A multi-barrel system allowed the application of different superfusion solutions through the same delivery pipette (Fig 3.5). Change from one reservoir solution to another was made faster by an additional suction needle in the dead space of the delivery pipette that served to evacuate the interior of the pipette quickly and enabled the speedy replacement by the superfusion solution of the next reservoir (Fig. 3.6). It was therefore possible to use more than one superfusion solution without changing the position and the size of the superfusion spot.

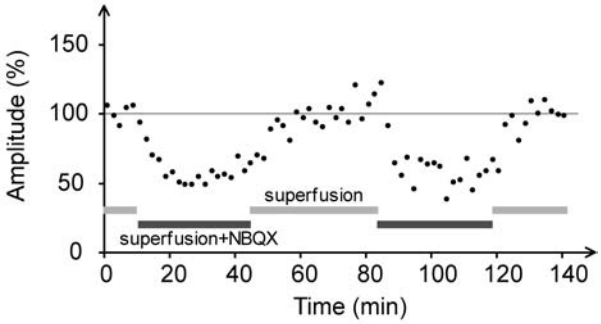


Figure 3.6 Rapid exchange of superfusion solutions. In this example experiment, normal superfusion solution and superfusion solution containing 10 µM NBQX (an AMPA receptor antagonist) were exchanged multiple times. This exchange took place within a few minutes. When NBQX was applied, fEPSP amplitude decreased. After washout, the signal reliably recovered to baseline.

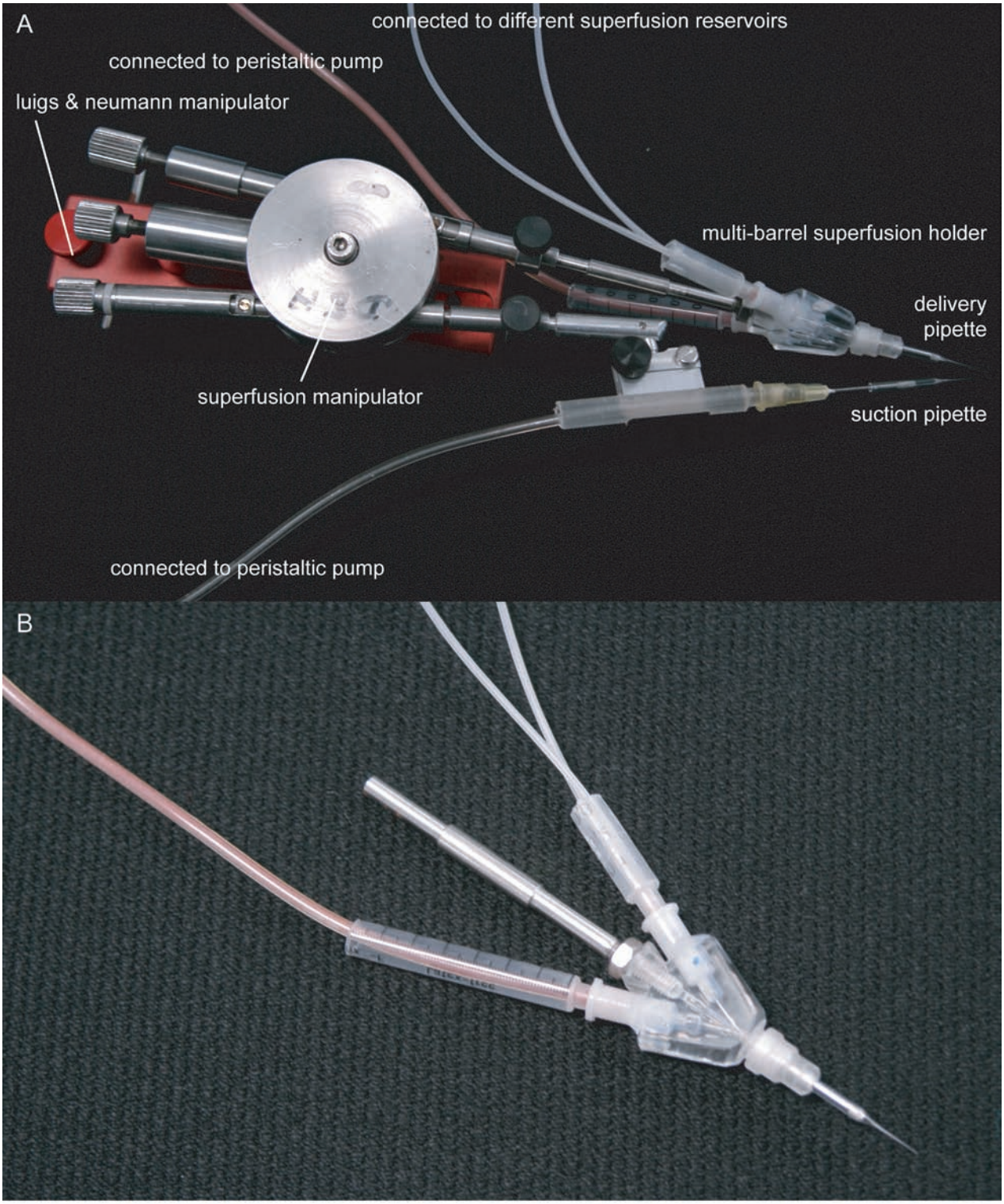


Figure 3.7 Superfusion device. (A) The superfusion pipettes were attached to a custom-made superfusion manipulator. This manipulator itself was mounted on a Luigs&Neumann manipulator. (B) Custom-made pipette holder for the delivery pipette, which allows to connect the delivery pipette to different superfusion reservoirs.

Name of dye	Type of dye	Concentration	Effect on neurons		Suitable?
			during application	after washout	
Bordeaux Red	food dye	1:1000	fEPSP decreases	recovery	no
Poinceau Red	food dye	1:3333	fEPSP decreases	partial recovery	no
Methylen Blue	vital dye	1:10 000	fEPSP disappears	no recovery	no
Fast Green	vital dye	1:40 000	fEPSP disappears	no recovery	no
Phenol Red	pH indicator	1:5000	fEPSP stable	stable	yes

Table 3.2 Different dyes tested for the superfusion. All dyes were bath-applied; the table indicates the effect of the lowest concentration examined, as assessed by extracellular recordings. Only phenol red proved suitable for my purposes.

3.4.2 Superfusion solution

In the superfusion solution an increased calcium concentration was used (Table 3.1). It is important to consider that the concentration in the superfusion solution is significantly diluted before it has reached the synaptic terminals deep in the tissue. Because a minimal concentration of 10mM Ca^{2+} was necessary to allow synaptic transmission in the superfusion spot during application of the blocking medium, the true concentration at synaptic sites was probably much lower.

The penetration depth of the superfusion solution was approximately 15 to 30µm, as assessed by electrophysiological measurements (see also Engert et al. 1997). While synaptic transmission outside the spot was blocked, the recording electrode inside the spot was gradually moved downwards. With increasing distance from the surface of the slice, the signal became smaller and eventually disappeared.

For visibility, the superfusion solution was supplemented with a dye. Surprisingly, the

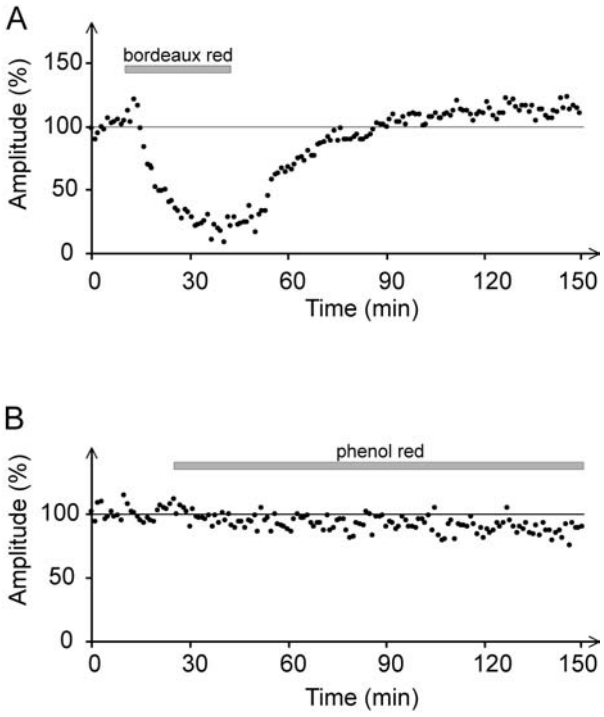


Figure 3.8 Different dyes tested for the superfusion. Both dyes were bath applied during the time indicated in the graph. (A) The food color bordeaux red (Brauns-Heitmann, 30mg/10ml) caused a decrease in fEPSP amplitude. (B) During application of phenol red (1:5000), the fEPSP remained stable.

food dye used in the previous superfusion studies (Engert and Bonhoeffer, 1997; 1999) turned out to be harmful for the cells under my recording conditions (Fig. 3.8A). When the dye was bath applied to the slice, the fEPSPs became dramatically smaller. Other food and vital dyes were tested and also had negative effects on the neurons (see Table 3.2). Finally, phenol red (Sigma-Aldrich, Germany), a pH indicator and as such standard component of neuronal cell culture media, proved suitable. A concentration of 0.5mM (= 1:5000) provided sufficient color intensity and had no adverse effects on the neurons in my conditions (Fig. 3.8B).

Conveniently, phenol red not only served to tint the superfusion solution but also allowed monitoring its pH. The superfusion reservoirs were continuously bubbled with carbogen. I used Teflon tubes with a small inner diameter to reduce the diffusion of gases and to keep the volume inside the tubes to a minimum (< 300 µl). When the superfusion was running, the pH remained constant. However, in the case of the multi-barrel superfusion, the superfusion solution from the closed reservoir changed its pH while standing still inside the tube. When the superfusion was then switched to this solution, it was made sure, that the internal suction was kept on long enough until the tube volume was evacuated and replenished by pH adjusted medium from the reservoir (usually within thirty seconds).

3.4.3 Time lapse study of the superfusion spot

The spot size was visually controlled during all experiments and no observable difference over time was detected. In order to document the stability of the spot, photographs of the slices and of the superfusion spot were taken. Pictures taken at different time points of an experiment documented that the spot remained stable over time (Fig. 3.9). Sometimes, spots slightly changed their shape. Possibly, this was due to swelling of tissue diminishing the distance between slice and superfusion pipettes. When this was accompanied by a change in the electrophysiological signal, the experiment was discarded.

Photographs immediately before and after changing from one superfusion solution to another demonstrated that it was possible to use more than one superfusion solution without altering the position and the size of the spot (Fig. 3.10). Accordingly, the amplitude of the electrophysiological signal was not altered by the exchange of superfusion solutions (see Table 3.3). The pictures shown were taken during the second series of the superfusion experiment.

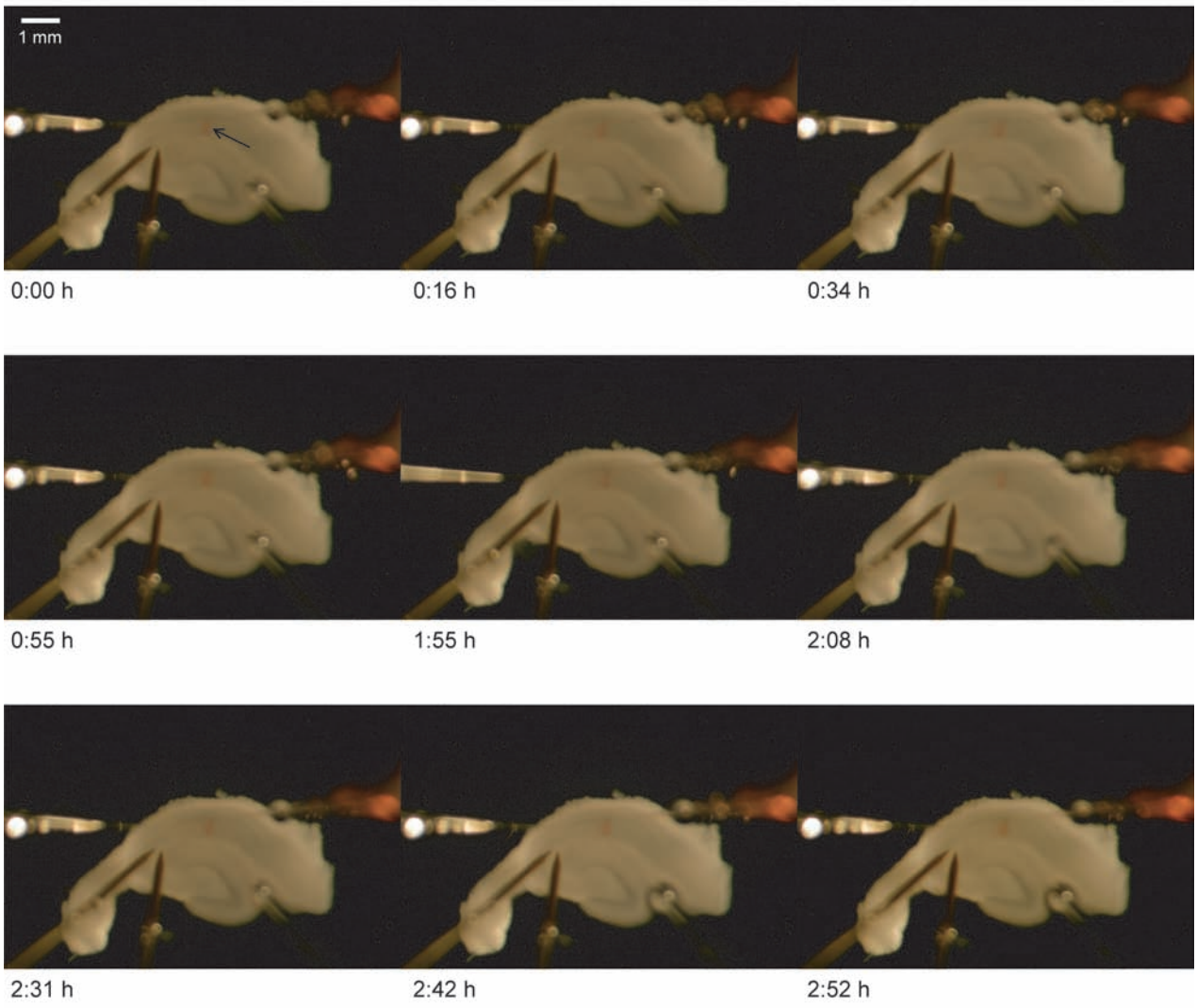


Figure 3.9 Time lapse study of the superfusion spot. Pictures taken at different time points of an experiment documented that the spot remained stable over time.

Figure 3.10 Superfusion exchange does not alter position and size of the superfusion spot. Photographs were taken before, during and after the exchange of superfusion solutions. During the change, the superfusion device was removed from the slice; therefore, no spot can be seen.

(fEPSP after exchange/fEPSP before exchange)%	Potentiation Experiments n=6	Control Experiments n=6
Exchange 1: normal => AP5 and verapamil	99 ± 2%	99 ± 3%
Exchange 2: AP5 and verapamil => normal	106 ± 3%	105 ± 6%

Table 3.3 Superfusion exchange does not alter fEPSP amplitude. In the second series of superfusion experiments, superfusion solutions were changed twice. First, normal superfusion solution was replaced by one containing AP5 and verapamil (exchange 1). Later, normal superfusion solution was reintroduced (exchange 2). To assess whether the exchange of superfusion solutions altered the size of the fEPSP, fEPSP amplitudes after the change were compared to baseline fEPSPs before the change. Amplitudes were averaged over a time period of five minutes. Neither the first nor the second exchange of superfusion solutions caused a significant change in fEPSP amplitude ($p<0.05$). Furthermore, there was no difference between test and control experiments ($p<0.05$).

3.5 Data acquisition and analysis

Electrophysiological recordings were performed using an Axoclamp 2B amplifier (Axon Instruments at Molecular Devices, USA) in bridge-mode. Data were digitally sampled at 10kHz for intracellular experiments and at 5kHz for all other conditions. Extracellular recordings were band-pass filtered between 1Hz and 1kHz; no filtering was applied to intracellular recordings. Data were stored and analyzed with custom made (Duck Products, Germany) LabView software (National Instruments, USA) on a PC.

For intracellularly recorded EPSPs and small fEPSPs in the superfusion experiments, an appropriate fit of the initial signal slope was difficult and consequently, the calculated slopes were highly variable. Therefore, maximal signal amplitudes was calculated which turned out to be the more robust and reliable parameter. For consistency, amplitude measurement was also used for the extracellular experiments, where slope calculation is more common in the literature. For the extracellular experiments, the results based on slope measurent were compared to the results based on amplitude measurement. As expected, there was no differnce in the general outcome not in the statistical significance levels of the experiments.

For all experiments, quantitative potentiation values were calculated averaging all trials over a time period of 10 minutes, relative to a baseline of 20 minutes (extracellular experiments) or 10 minutes (all others) prior to the (first) tetanus. I.e. ,“LTP after one hour” refers to the average potentiation value 51-60 minutes after the (last) tetanus. During intracellular recordings, some neurons occasionally fired an action potential in response to Schaffer collateral stimulation. These trials were excluded for the calculation of the average potentiation value. In the first superfusion series (Fig. 4.15), not all of the eight potentiation or eight control experiments lasted 60 minutes post tetanus. For consistency, the same time window of 51-60 minutes was applied for calculating the average, which means that the n is lower (6 respectively 5 out of the initial 8 experiments).

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis

was carried out using Excel (Microsoft, USA) or STATISTICA software (StatSoft, USA). Student's t-tests were used to analyze differences in physiological parameters. T-tests were always performed two-tailed; the type of t-test was chosen according to the specific test situation (paired, two-sample equal variance or two-sample unequal variance). F-tests were used beforehand to determine whether the variances of two experimental samples were significantly different. For all statistical tests, the significance value was set at $p<0.05$.

4 Results

To address the question whether neurons possess a homeostatic mechanism that regulates LTP induction at hitherto non-potentiated synapses I performed electrophysiological experiments in rat hippocampal slices using classical NMDA-receptor dependent CA3-CA1 LTP (Fig. 3.1). In the first set of experiments, two independent Schaffer collateral pathways were stimulated, to test the following homeostatic hypotheses:

Null hypothesis:

Pathways are fully independent. LTP induction on one pathway does not affect LTP induction on another pathway.

Strong homeostatic hypothesis:

LTP induction on one pathway blocks all subsequent LTP on another pathway.

Weak homeostatic hypothesis:

LTP induction on one pathway reduces subsequent LTP on another pathway.

4.1 Extracellular recordings

4.1.1 Probing the homeostatic hypothesis with potentiated synapses

In a first set of experiments, I wanted to examine whether potentiation in one Schaffer collateral pathway might prevent or diminish further potentiation of neighboring synapses from an independent set of Schaffer collateral axons. To this end, fEPSPs were measured extracellularly in the CA1 region while stimuli were elicited in the CA3 region (see Fig. 3.1). A tetanic stimulus was used to induce LTP on one pathway and one hour after the tetanus, 137 ± 5% LTP was expressed (Fig. 4.1A, n=9, in seven rats, see Methods for details about data analysis). One hour after LTP induction in the first pathway, a second, independent pathway ('test pathway') was probed for its ability to undergo LTP with the same stimulus. Classically, the principle of input specificity should result in LTP on one pathway being independent of that on another pathway. However, if the total amount of potentiation within a population of synapses was limited, the homeostatic hypothesis would predict that previous potentiation should affect LTP induction on another pathway. LTP on a second pathway might be blocked completely (strong homeostatic hypothesis) or at least reduced (weak homeostatic hypothesis). Experimentally I found that this was not the case, as normal LTP was observed in the test pathway as well (142 ± 9% one hour after tetanus, n=9, in seven rats). The amount of LTP in the test pathway was not significantly different from that in the first pathway (p=0.54), which already suggested that the first LTP did not impair induction of LTP in the test pathway. Additionally, control experiments were performed, where the initial LTP induction did not take place, and only one tetanus was given after 80 minutes recording time (Fig 4.1 B).

According to the homeostatic hypothesis, LTP in the control slices should be higher than that of the test pathways, but this was not observed. In contrast, LTP in the control experiments was even slightly lower than the test pathway LTP, but one hour after the tetanus, the difference was not statistically significant

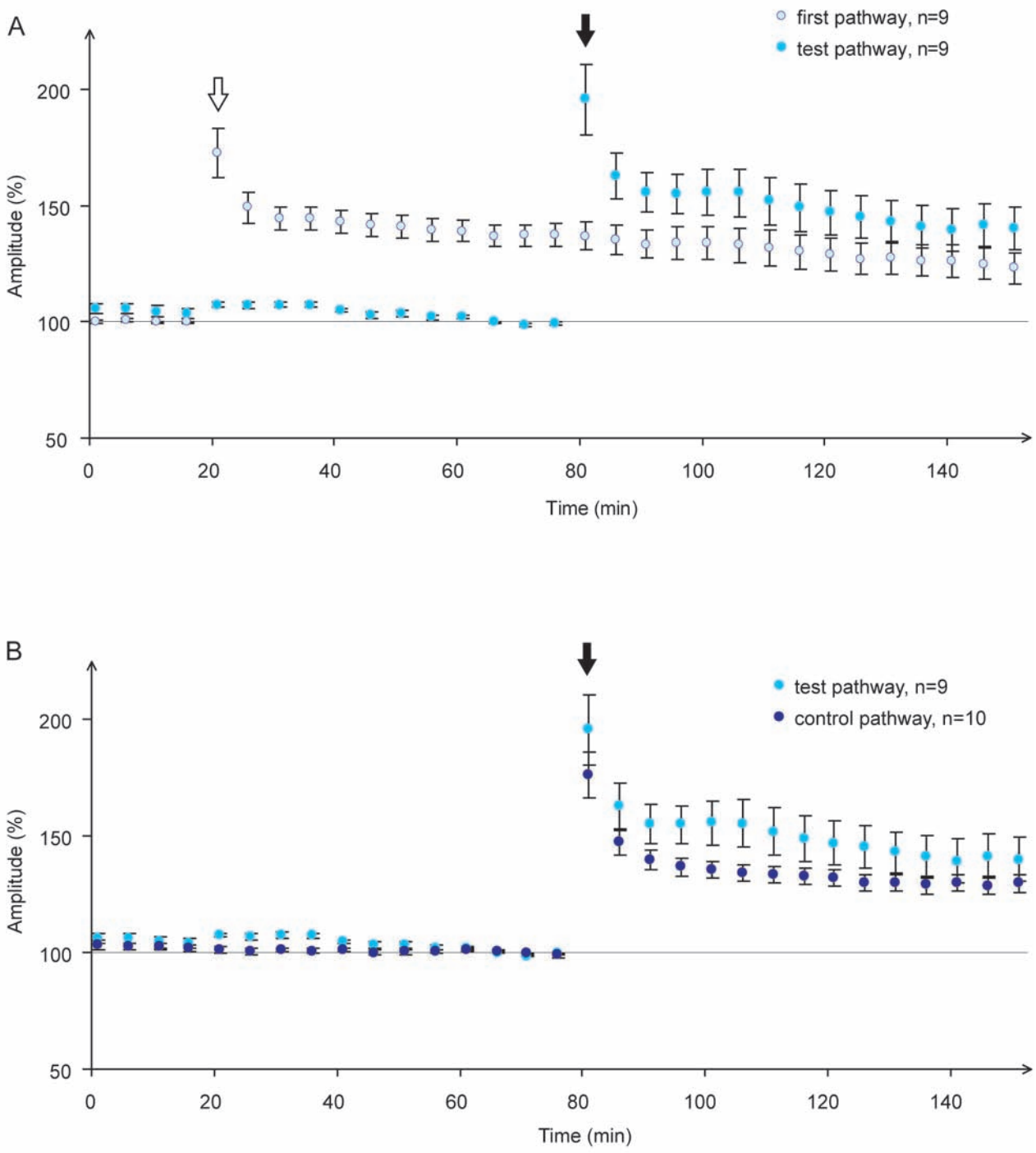


Figure 4.1 LTP on one Schaffer collateral pathway did not block or reduce LTP on another pathway in the same slice. (A) Tetanic stimulation on one pathway induced LTP in that pathway (137 ± 5% one hour after tetanus, n=9). One hour later, a second independent pathway in the same slice received a single tetanus to probe its capability to undergo LTP. The second pathway showed normal LTP as well (142 ± 9% one hour after tetanus, n=9). (B) The amount of LTP in the second pathway was not significantly different from that in the first pathway and was no smaller than that seen in control slices without previous LTP induction (129 ± 3% one hour after tetanus, n=10, $p>0.1$). (A, B) Potentiation values were calculated relative to a baseline of 20 minutes prior to the tetanus (100 pulses, 100Hz). White arrow: tetanus to the first pathway, black arrow: tetanus to the test or control pathway.

(LTP in control slices:129 ± 3%, n=10, in six rats; LTP in the test pathways: 142 ± 9% one hour after tetanus, n=9, in seven rats; p=0.22).

In these experiments, LTP induction on one pathway neither blocked nor reduced LTP on a second pathway. Therefore, both the strong and the weak form of the homeostatic hypothesis were refuted. However, LTP in the first pathway was not particularly high and decayed rather rapidly (127 ± 7%, 2 hours after tetanus, n=9, in seven rats, Fig. 4.1). Probably, only an early, protein-synthesis independent form of LTP had been induced which may not have caused the necessity of homeostatic intervention.

In view of that, I modified my homeostatic hypothesis. The initial experiments demonstrated that normal, transient LTP did not interfere with further LTP induction at other synaptic sites, but I speculated that saturated and long-lasting LTP might well do. Accordingly, I adapted the experimental paradigm to induce saturated LTP in the first pathway, which was long-lasting (six hours) and extended into the protein-synthesis dependent time range.

4.1.2 Probing the homeostatic hypothesis with saturated synapses

I used multiple tetanization of one pathway to induce saturated and long-lasting LTP. 'Saturation' was operationally defined as the inability to induce further LTP on that specific pathway with the same pattern of tetanization. During experiments, this definition was implemented by the following three rules:

1. Apply at least three episodes of tetanic stimulation with an inter-tetanus interval of 10 minutes.
2. Continue with tetanic stimulation in ten-minute intervals until the increase in fEPSP amplitude from the $(n-1)^{th}$ tetanus to the n^{th} tetanus is less than ten percentage points.
3. Discard the experiment if the potentiation has fallen below 130% one hour after the last tetanus.

For the summary analysis, all experiments were aligned to the time point of the last tetanus. The saturation criteria ensured that the first, saturated pathway exhibited indeed strong and long-lasting LTP (156 ± 6% one hour and 144 ± 9% six hours after the last tetanus, n=12, in twelve rats, Fig 4.2A, the difference between the two timepoints was not significant, p=0.05). As before, a second independent pathway ('test pathway') was tested for its capability to undergo LTP one hour later. The modified homeostatic hypothesis in its strong form would predict that saturation of LTP on one pathway that induces L-LTP blocks all LTP on an independent input. The weaker version of the hypothesis would claim that early LTP on the test pathway might be left intact and that only L-LTP might be affected by previous saturation. To differentiate between early and late effects, long-term experiments were performed. The strong hypothesis could be refuted quite clearly: in the test pathway, robust LTP was observed, which was no smaller than that observed in control slices without previous saturation (test pathways: 138 ± 5% one hour after LTP induction, n=12, in twelve rats; control pathways: 136 ± 6% one hour after LTP induction, n=10, in ten rats, p=0.80; Fig 4.2B).

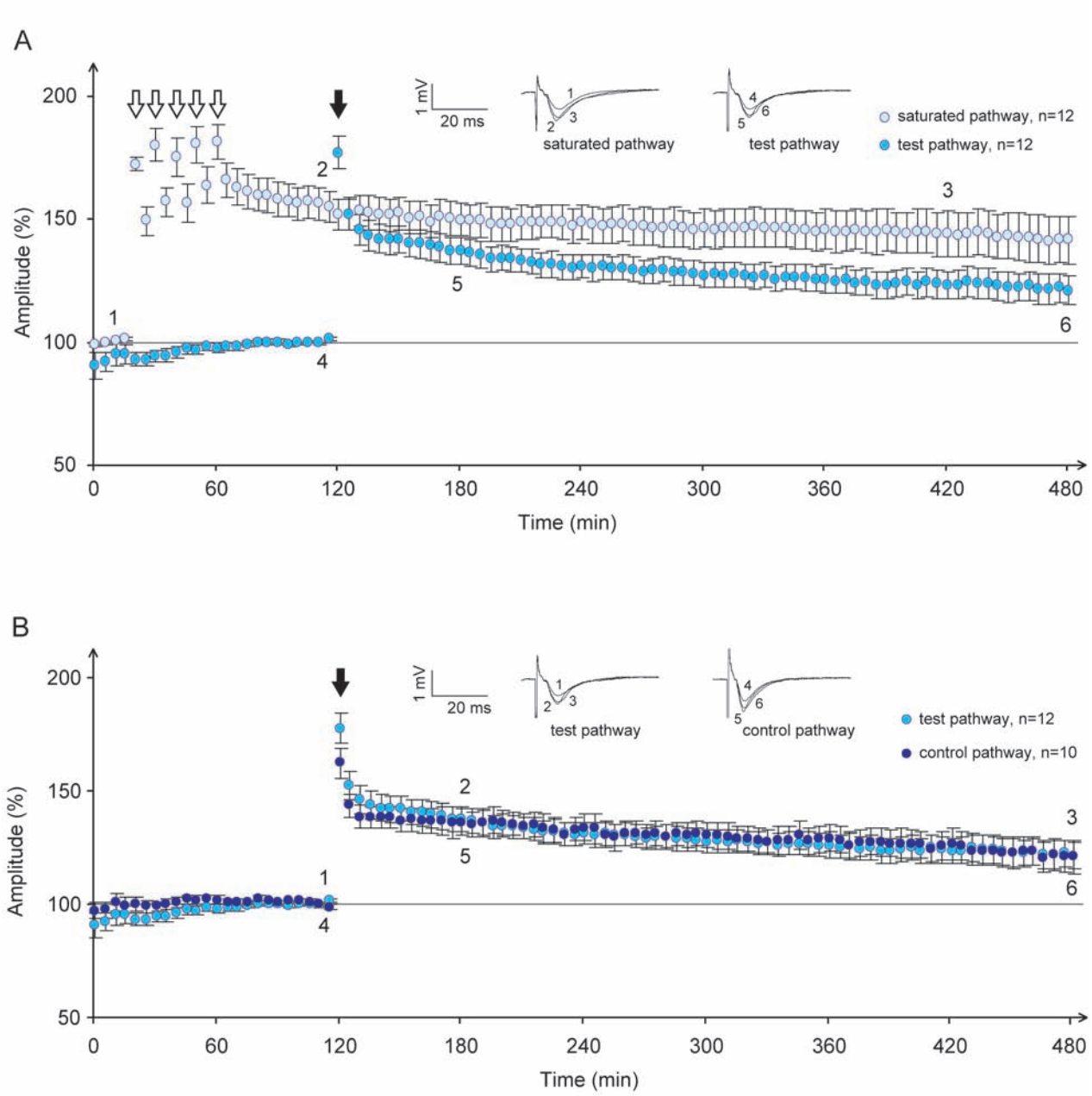


Figure 4.2 Saturated LTP on one Schaffer collateral pathway did not block or reduce LTP on another pathway in the same slice. (A) Multiple tetanization of one Schaffer collateral pathway induced saturated and long-lasting LTP ('saturated pathway', 156 ± 6% one hour and 144 ± 9% six hours after the last tetanus, n=12). One hour later, a second independent pathway in the same slice ('test pathway') received a single tetanus to probe its capability to undergo LTP. The test pathway showed robust and long-lasting LTP as well (138 ± 5% one hour and 122 ± 6% six hours after LTP induction, n=12). (B) The amount of LTP in the test pathways was not different from LTP observed in control slices without previous saturation ('control pathways', 136 ± 6% one hour and 121 ± 7% six hours after LTP induction, n=10, $p > 0.1$). (A, B) Potentiation values were calculated relative to a baseline of ten minutes prior to the (first) tetanus. Representative fEPSPs from a saturation and a control experiment were taken before and after LTP induction at the time points indicated in the graphs (averaged over five consecutive sweeps). White arrow: tetanus to the saturated pathway, black arrow: tetanus to the test or control pathway. Tetanus: 100 pulses, 100Hz.

	Time point	Stimulation electrode proximal	distal	p-value
Saturated pathways	1 h after tetanus	158 ± 9%, n=7	152 ± 10%, n=5	0.69
	6 h after tetanus	147 ± 9%, n=7	141 ± 19%, n=5	0.79
Test pathways	1 h after tetanus	129 ± 7%, n=5	144 ± 7%, n=7	0.17
	6 h after tetanus	117 ± 11%, n=5	125 ± 8%, n=7	0.55

Table 4.1 The position of the stimulation electrode has no influence on the amount of LTP induced. Baseline: 20 minutes prior to the tetanus.

Also the weaker prediction was falsified, because the maintenance phase of LTP was not affected either (six hours after tetanus: 122 ± 6% in the test pathways, 121 ± 7% in the control pathways, p=0.96; Fig 4.2B). Although at this point test LTP was considerably lower than that on the saturated pathway (because I had used only one tetanus to 'probe' for the capability to undergo potentiation), there was no difference between the (equivalent) test and control pathways.

Does the position of the stimulation electrode, e.g. more proximal or more distal to the cell layer, have an influence on the amount of LTP induced through that electrode? To avoid any possible bias, I alternated between experiments which of the two stimulating electrodes was used for the saturated pathway and which for the test pathway. The amount of LTP induced through the electrode proximal to the cell layer was not statistically different to the amount of LTP induced through the distal electrode (see Table 4.1).

		Saturation Experiments grouped by number of tetani			Saturation Experiments summary		Control Experiments summary
Number of tetani on saturated pathway		3	4	5	3, 4 or 5		-
Number of experiments		n=5	n=4	n=3	n=12		n=10
LTP (%) 1h post tetanus	sat.	139±9[a]	162±7	175±2[a]	156±6[d]		-
	test	126±7[b]	146±11	147±2[b]	138±5[d]	control	136±6
6h post tetanus	sat.	130±17	141±8[c]	174±6[c]	144±9[e]		-
	test	116±8[d]	115±14	140±3[d]	122±6[e]	control	121±7

Table 4.2 Amount of LTP in the saturated, the control and the test pathways. Baseline: 20 minutes prior to tetanus. Letters a-e indicate that there was a significant difference between the respective pair of values ($p<0.05$).

Three out of fifteen saturation experiments were discarded according to rule three of the saturation criterion (potentiation <130% one hour after tetanus). Of the twelve experiments accepted for summary analysis, five experiments received three tetani, four experiments received four tetani and three experiments received five tetani in their first, i.e. the saturated pathway (see Table 4.2). One hour post tetanus, the three-tetani-experiments showed 139 ± 9% LTP, the four-tetani experiments showed 162 ± 7% and the five-tetani-experiments showed 175 ± 2% LTP in their saturated pathways. The respective test pathways exhibited 126 ± 7%, 146 ± 11% or 147 ± 2% LTP. The difference in the amount of potentiation between five and three tetani was statistically significant for the saturated pathways as well as the control pathways ($p<0.05$). The difference between five and four tetani or four and three tetani was not statistically significant ($p>0.10$).

Six hours after tetanus, the three-tetani-experiments showed 130 ± 17% LTP, the 4-tetani experiments showed 141 ± 8% and the 5-tetani-experiments showed 174 ± 6% LTP. The respective test pathways exhibited 116 ± 8%, 115 ± 14% or 140 ± 3% LTP. For the test pathways, the difference in the amount of potentiation between five and three tetani was statistically significant ($p<0.05$), but not for the saturated pathways. The saturated pathways, in contrast, exhibited a significant difference between four and five tetani ($p<0.05$).

In summary, those slices that were saturated already after three tetani exhibited the lowest amount of potentiation; and those slices that were saturated only after five tetani showed the highest amount of potentiation. Interestingly, this was true for both saturated and test pathways. Comparing the different subclasses revealed that high potentiation in the saturated pathway entailed high potentiation in the test pathway. This was corroborated by a within-experiment comparison: The amount of potentiation in the saturated pathway was plotted against the amount of potentiation in the test pathway (Fig. 4.3).

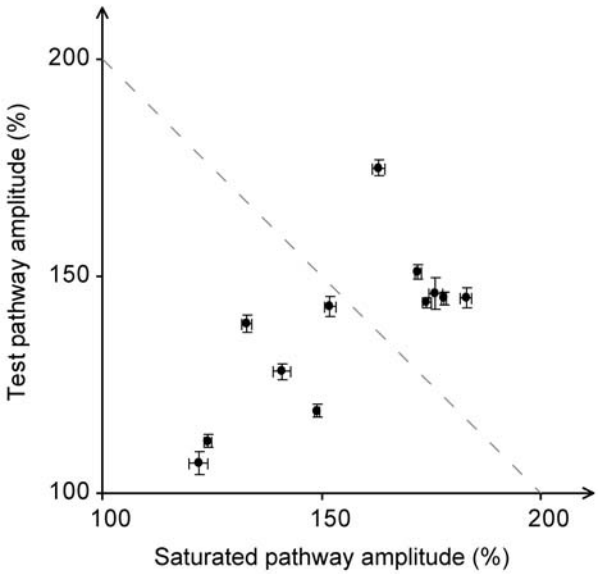


Figure 4.3 Correlation analysis of the extracellular data. The amount of LTP in the saturated pathway is plotted against the amount of potentiation in the test pathway one hour after the (last) tetanus in each pathway (correlation coefficient $r^2=0.51$. $p<0.01$, $n=12$). Two experiments had less than 130% LTP (measured as change in signal amplitude) in the saturated pathway one hour after the last tetanus. Nevertheless, these experiments were accepted for the summary analysis, because their signal slope was above 130%. Originally, fEPSP slope was the relevant parameter for the extracellular recordings, which the saturation rules based upon. However, it turned out later that measuring slope was unsuitable for the intracellular and superfusion experiments. For these experiments, amplitude was the appropriate parameter. To be consistent, analysis of the extracellular experiments was therefore changed from slope to amplitude. There was no qualitative difference between slope and amplitude analysis of the extracellular experiments. Dotted line: expected anti-correlation.

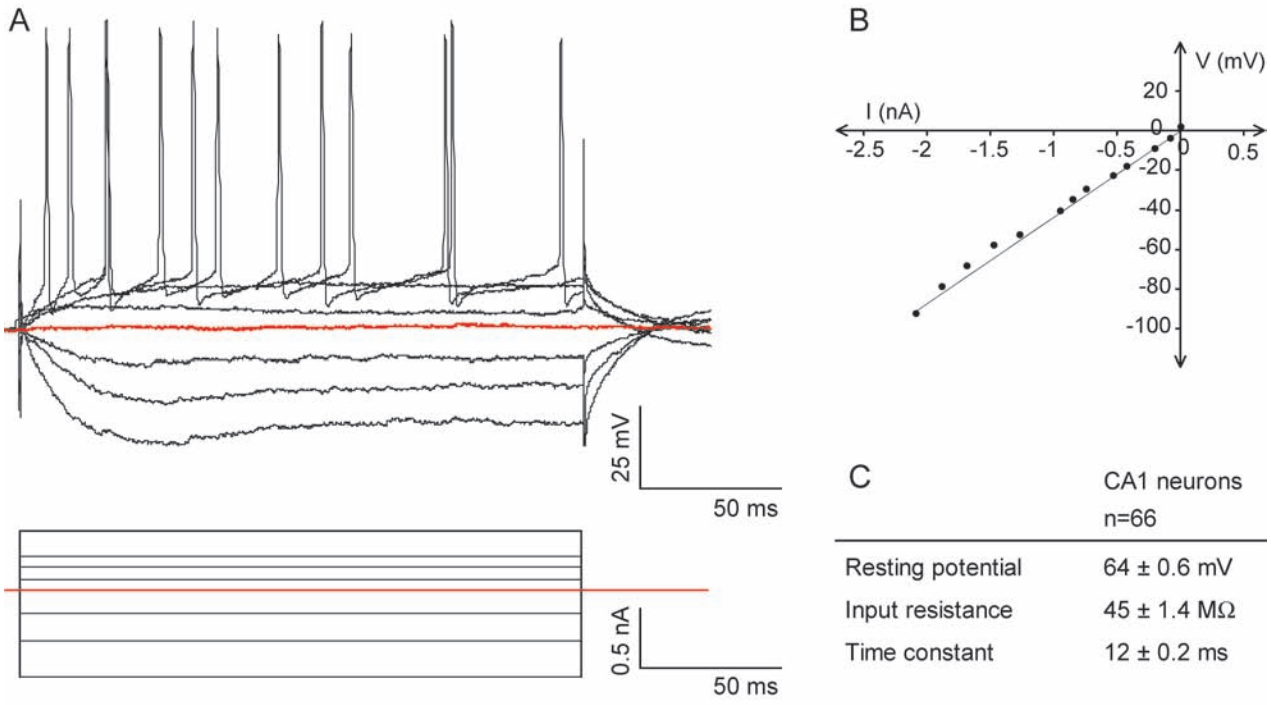
Experiments exhibiting high LTP in the saturated pathway also showed high LTP in the test pathway (correlation coefficient

r^2=0.51, p<0.01). However, if a homeostatic mechanism had been active, one would expect to see the opposite: high LTP on one pathway should block or reduce LTP induction on another pathway, to keep overall potentiation within reasonable limits.

Although at first glance, these observations argue against any homeostatic regulation of synaptic efficacy, they are not yet conclusive. In extracellular recordings, the two populations of CA1 neurons contributing to the field potentials activated by the two stimulation pathways might not fully overlap. A potential homeostatic effect might therefore remain undetected. I decided to address this issue with intracellular recordings from single CA1 neurons.

4.2 Probing the homeostatic hypothesis on a single neuron

In a new set of experiments, I performed single-cell recordings of CA1 neurons to get a clear-cut answer as to whether the applied stimuli lead to homeostatic response. With regard to the stimulation the experimental paradigm was very similar to that used for the extracellular recordings. Before an actual experiment was started, basic electrophysiological properties of the recorded neuron were tested. A series of hyperpolarizing and depolarizing step currents were injected through the recording electrode while changes in membrane potential were monitored (Fig. 4.4). Thereby, passive membrane properties of 66 neurons could be measured. On average, CA1 neurons had a resting potential of 64 ± 0.6mV, an input resistance of 45 ± 1.4MΩ and a time constant



C

	CA1 neurons n=66
Resting potential	64 ± 0.6 mV
Input resistance	45 ± 1.4 MΩ
Time constant	12 ± 0.2 ms

Figure 4.4 Electrophysiological properties of CA1 neurons. (A) A series of hyperpolarizing and depolarising step currents were injected through the recording electrode while changes in membrane potential were monitored. Red line: no current injection. (B) The current-voltage relation (IV-curve) was used to determine the membrane resistance. (C) Passive membrane properties of 66 hippocampal CA1 neurons.

of 12 ± 0.2ms (see Fig 4.4). These values are in accordance to what has been reported in the literature for pyramidal neurons in the hippocampus (Mason, 1992).

A particular concern was that LTP induction in the test pathway took only place very late in the experiment, more than 1 ½ hours after the penetration of the neuron with the recording electrode. I worried that some other factors might have deteriorated unnoticeably which might compromise LTP induction that late in time. Impaired LTP on the test pathway would then be erroneously attributed to the prior saturating stimulus. Therefore, control experiments were performed, where a tetanus was given after 90 minutes recording time without previous saturation (for an example, see Fig. 4.5). Although the potentiation in the control experiments was not very high (109 ± 6% one hour after tetanus, n=5, in five rats), it was significantly different to baseline values (p=0.22). Thus, the control experiments showed that LTP induction was still possible after long recording times and thereby established that the single cell approach was suitable.

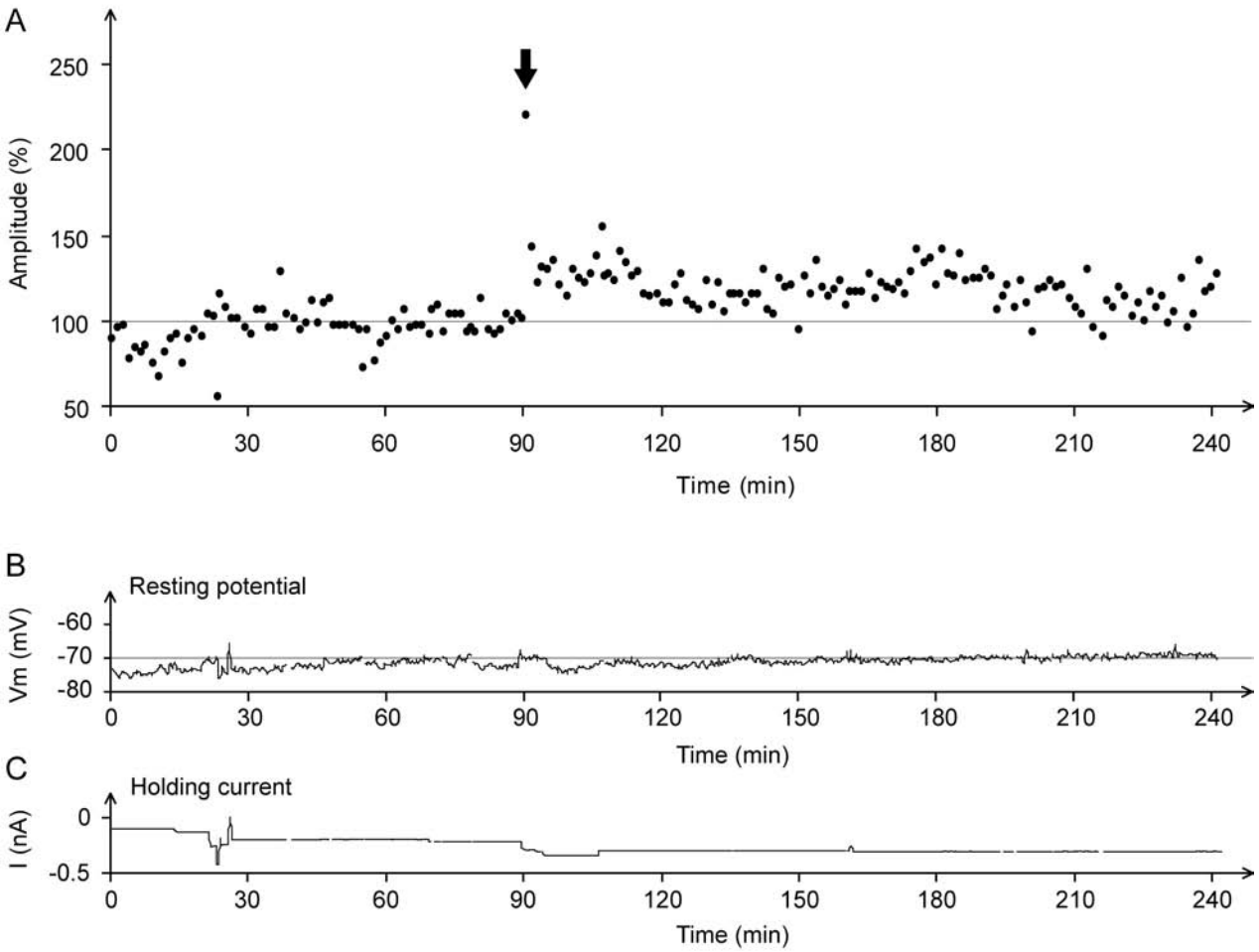


Figure 4.5 Example of an intracellular control experiments. Potentiation values were calculated relative to a baseline of ten minutes prior to the tetanus. Arrow: tetanus (100 pulses, 100Hz). (B) Resting membrane potential of the neuron and (C) holding current were monitored throughout.

For the intracellular saturation experiments, the stimulation pattern was very similar to that used for extracellular recordings. Following a short baseline, three successive tetanic trains were applied to saturate LTP on the first pathway. Like in the first series of experiments, the test pathway received a single 'probe' tetanus one hour after the last of the saturating tetani. The saturation criteria from the extracellular experiments were slightly modified to match the technical challenges of long-term intracellular recordings.

Rule two of the saturation definition was not applied: the first pathway always received three tetani but not more. The extracellular experiments have shown that for the largest fraction of experiments, three tetani were sufficient to induce saturated LTP. Furthermore, experimental time could be saved which was especially important considering the long recording time of minimally three hours. In rule three, the threshold for stopping or discarding an experiment was set to 125% LTP one hour after tetanus rather than 130%.

The single cell approach can now provide conclusive answers as to whether the applied stimuli entail a homeostatic response of the neuron. Unexpectedly, I observed that, despite apparently saturating LTP in the first pathway, CA1 neurons still exhibited additional potentiation in response to a tetanus in the second pathway.

Two example experiments are shown in Fig. 4.6 and Fig. 4.7. In the first experiment (Fig. 4.6), EPSP amplitude of the saturated pathway was markedly increased to double its baseline value following repeated tetanic stimulation. After the single 'probe' tetanus, the test pathway showed robust LTP as well. Sporadically, an action potential was elicited, indicated in the graph by a vertical bar. As for all experiments, resting membrane potential and holding current were continuously monitored.

In the second example, (Fig 4.7), multiple tetanization caused such a strong potentiation in the saturated pathway that every test stimulus elicited an action potential for the first hour post-tetanus. Baseline amplitudes in the second pathway did not change upon potentiation of the first. When the second pathway was tetanized one hour later, it showed clear potentiation, too, so that test stimulation evoked action potentials as well. Occasionally elicited subthreshold EPSPs were clearly above baseline in both pathways, indicating that tetanization had indeed caused synaptic strengthening rather than a change in the firing threshold. The spiking rate of this experiment was unusually high, but it was a striking observation that despite the very strong synaptic potentiation on the first pathway, the test pathway still could be strengthened in a way that it exhibited action potentials as well. Although it is not more than a single example, the latter experiment with its pronounced potentiation in both pathways provides already considerable counter-evidence against homeostatic regulation following this specific pattern of stimulation.

For a quantitative summary analysis, average potentiation values of each experiment were calculated for a time bin of ten minutes one hour after the (last) tetanus of the respective pathway. Trials where an action potential was fired were excluded, it was ensured that at least five trials contributed to the average value. If during the relevant time window, no or not enough subthreshold EPSPs were recorded, consecutive EPSPs at a later time-point were taken into the calculation.

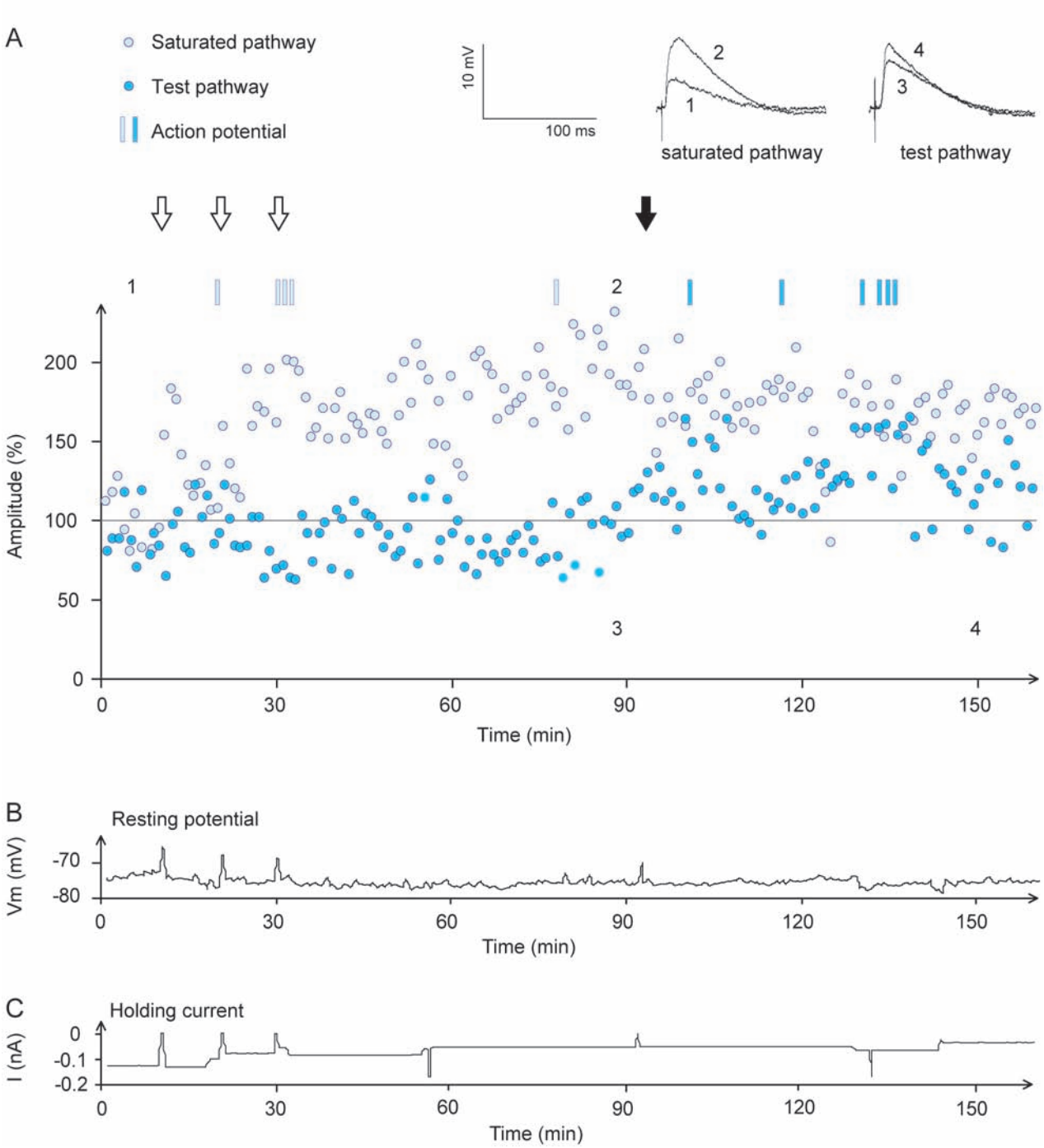


Figure 4.6 Example of an intracellular saturation experiment. Potentiation values were calculated relative to a baseline of ten minutes prior to the tetanus. White arrow: tetanus to the saturated pathway, black arrow: tetanus to the test or control pathway. Tetanus: 100 pulses, 100Hz.. Representative fEPSPs averaged from five consecutive stimuli were taken before and after LTP induction at the time points specified in the graph. (B) Resting membrane potential of the neuron and (C) holding current were monitored throughout.

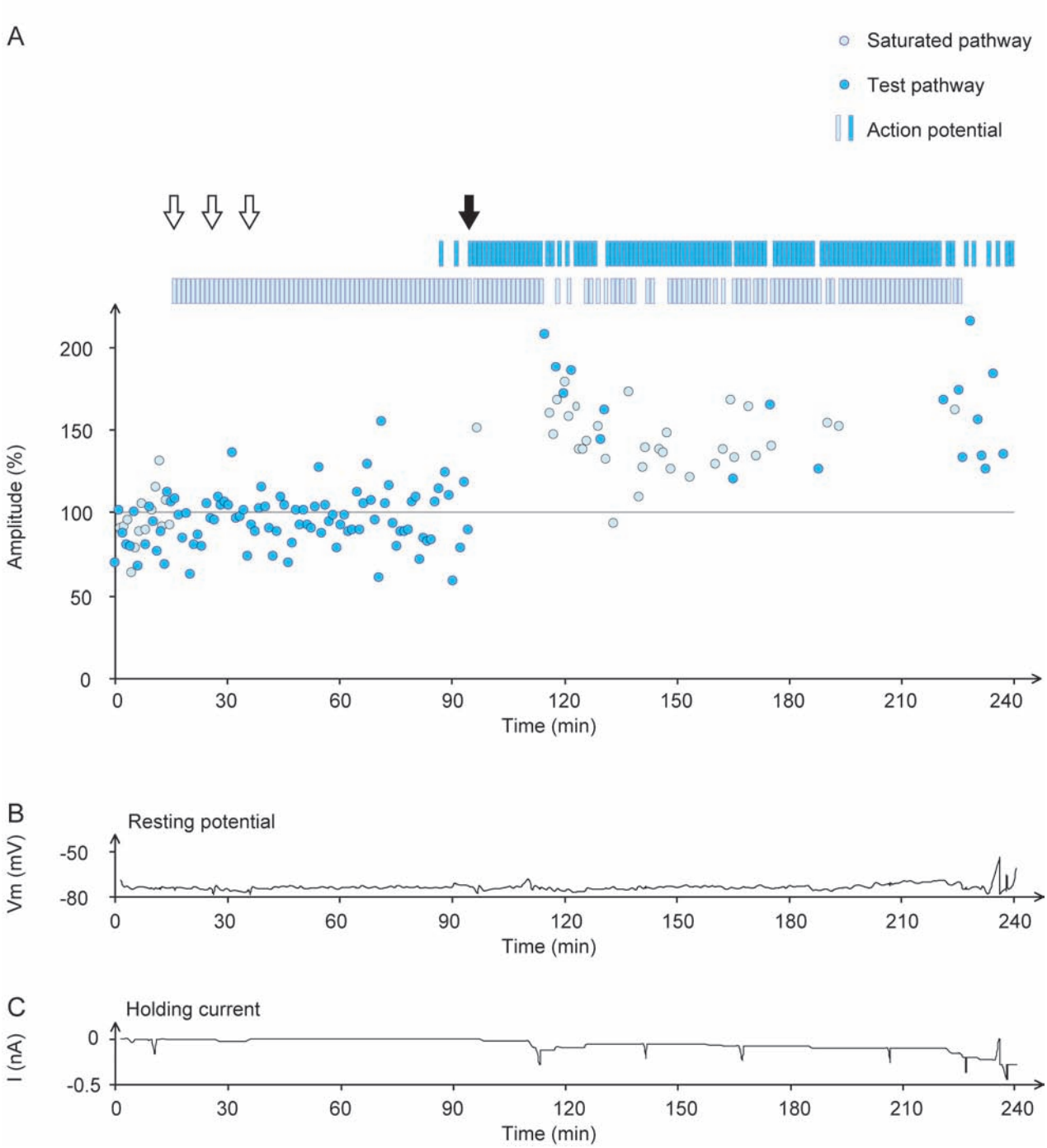


Figure 4.7 Example of an intracellular saturation experiment. Potentiation values were calculated relative to a baseline of ten minutes prior to the tetanus. White arrow: tetanus to the saturated pathway, black arrow: tetanus to the test or control pathway. Tetanus: 100 pulses, 100Hz.. (B) Resting membrane potential of the neuron and (C) holding current were monitored throughout.

Average potentiation in the saturated pathways was 151 ± 8% and 124 ± 7% in the test pathways (one hour after the respective tetanus, n=8, in eight rats). If a neuron limits its total amount of potentiation, a negative correlation should be expected such that the higher the LTP in the saturated pathway, the lower the LTP in the test pathway. However, when LTP in the saturated pathway was plotted against LTP in the test pathway, there was no negative correlation whatsoever (correlation coefficient r^2=0.08, p=0.48, n=8, Fig. 4.8). If anything, a weak positive correlation was observed, which is however not statistically significant because of two outlier data points. The general tendency was that neurons which exhibited high potentiation in the saturated pathway also showed strong potentiation in the test pathway.

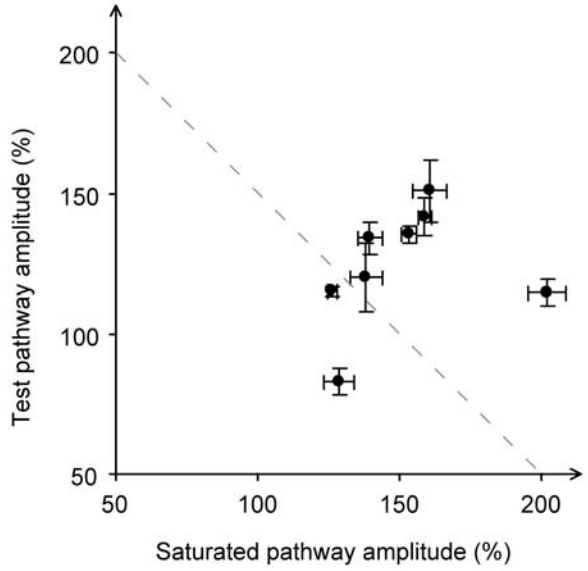


Figure 4.8 Correlation analysis of the intracellular data. The amount of LTP in the saturated pathway is plotted against the amount of potentiation in the test pathway one hour after the (last) tetanus in each pathway (correlation coefficient r^2=0.08. p=0.48, n=8). Dotted line: expected anti-correlation.

Homeostatic theory would predict that control experiments showed higher LTP than the test pathways of the saturation experiments, but this was not the case. On average, LTP in the control experiments was 109 ± 6% (n=5) one hour after tetanus; test pathway LTP of the saturation experiments was 124 ± 7% (n=8). Surprisingly, LTP in the test pathways appeared slightly higher than that of the control experiments, but the difference was not statistically significant.

4.3 Probing the homeostatic hypothesis by potentiating a large number of synapses

Neither the extracellular nor the intracellular recordings provided evidence for homeostatic downregulation of LTP, presumably, because the potentiation regime used so far did not exceed a crucial threshold for activating a homeostatic mechanism Operational saturation of the first pathway was intended to induce maximal potentiation in those synaptic contacts that were activated by one of the extracellular stimulation electrodes in the Schaffer collaterals. Although individual synapses were highly potentiated, it may be that the proportion of potentiated synapses on the postsynaptic neuron was too low to trigger homeostatic regulation of LTP.

Accordingly, in a new series of experiments, I turned to an alternative protocol for inducing LTP, which should result in a much larger proportion of synapses on individual neurons being potentiated.

4.3.1 Chemical Potentiation as a means to stimulate a large proportion of synapses

Instead of electrical tetanization, I used chemical potentiation to achieve widespread synaptic strengthening. Chemical LTP can be induced by a number of different pharmacological cocktails (e.g. Lu, Wu and Gean, 1999; Bortolotto and Collingridge, 1992; 1993; 1995; O'Leary and Connor, 1997). The potentiation medium I used contained increased potassium and calcium, reduced magnesium and 25mM of the potassium-channel blocker TEA (Aniksztejn and Ben-Ari, 1991; Hosokawa et al., 1995; see Methods). Experimental evidence from a number of studies suggests the following mechanism for TEA LTP: neurons become depolarized and the release of glutamate is transiently enhanced. Although different authors come up with slightly different results, overall it seems that both NMDA receptors and voltage-dependent calcium channels (VDCCs) are activated leading to increased postsynaptic calcium influx (Huang and Malenka, 1993; Hanse and Gustafsson, 1994; Huber et al., 1995; Song et al., 2001).

It has been reported by a number of laboratories that chemical and electrical LTP have similar properties (e.g. Hosokawa et al., 1995; Song et al., 2001). Nevertheless, it was important to confirm the equivalence of electrical and chemical potentiation in my recording conditions. Otherwise, one could not distinguish whether any potential effect is due to homeostatic regulation or just an artifact of the chemical potentiation. Therefore, I carried out a number of experiments to characterize the time course and pharmacological sensitivity of chemical LTP.

Time course of chemical LTP

Extracellular recordings were performed following standard procedure. A ten-minute bath application of potentiation medium reliably induced chemical LTP (177 ± 12% one hour after LTP induction, n=14, in four rats, Fig. 4.9A). The time-course of potentiation was different to tetanus LTP and showed two characteristic "humps". When the potentiation medium reached the recording chamber (approximately after two minutes, flow rate: 1ml/min), fEPSP shape and amplitude immediately changed. In most experiments, amplitudes increased, but sometimes, although the signal shape changed, amplitudes stayed constant or decreased. Field EPSP amplitudes increased a second time when the potentiation medium was washed out. Thereafter, signal amplitudes were continuously rising for 10 to 20 minutes, followed by a decrease until a stable plateau was reached 40 minutes after application of the potentiation medium. Similar to tetanus LTP, expression of chemical LTP was long-lasting (137 ± 11% six hours after LTP induction, n=5, in three rats, Fig. 4.9B).

Pharmacological sensitivity of chemical LTP

If chemical LTP acts through the same signaling pathways as conventional tetanic LTP, pharmacological blockade of these pathways known to interfere with electrical LTP should impair chemical potentiation as well. In fact, the NMDA-receptor antagonist AP5 (50μM) substantially reduced chemical LTP (123 ± 5% one hour after LTP induction (n=18, in three rats, Fig. 4.10A). However, chemical potentiation was not completely blocked. This observation confirmed prior findings that chemical LTP does require NMDA receptor

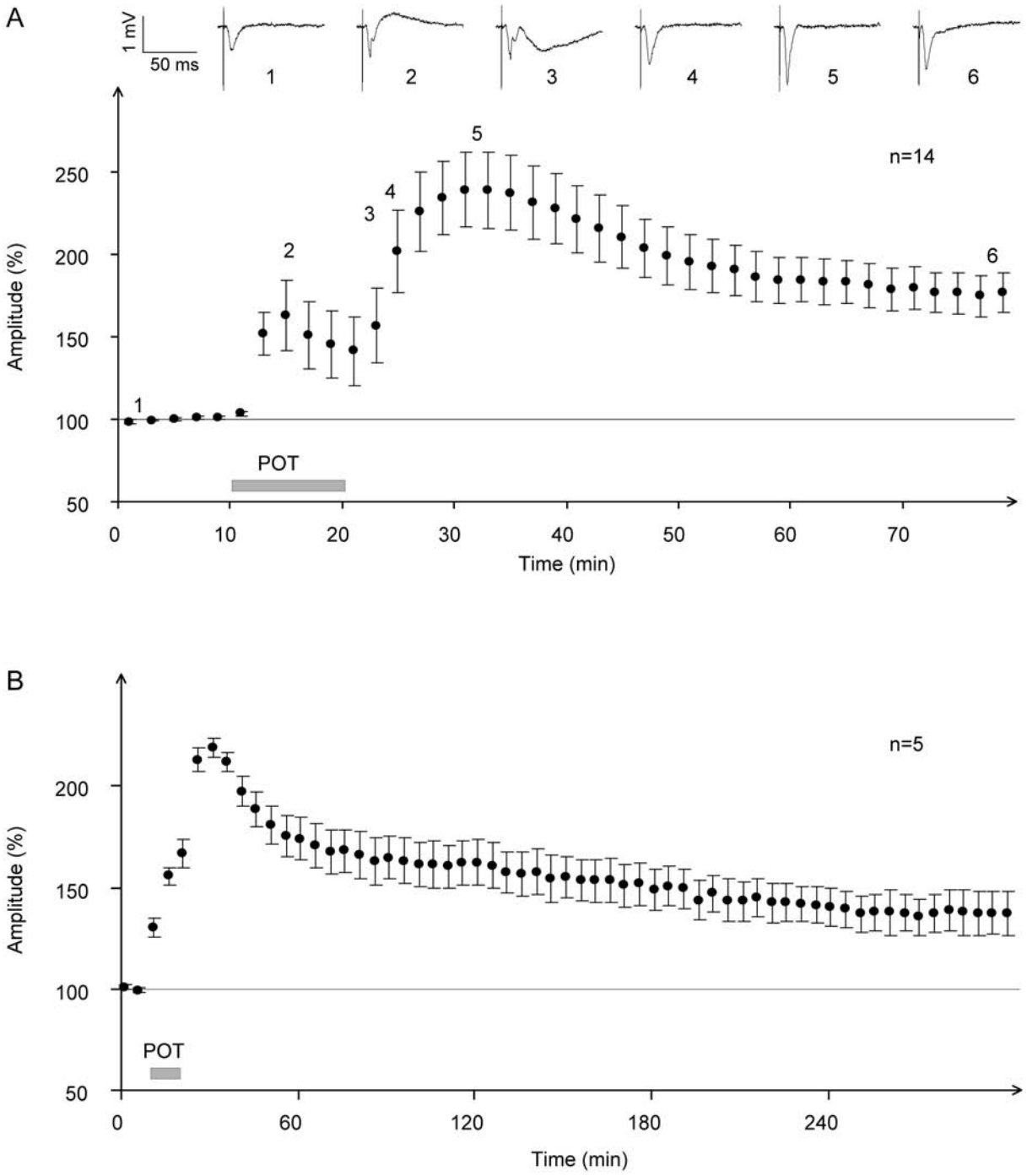


Figure 4.9 Time course of chemical potentiation. (A) A ten-minute bath application of potentiation medium (grey bar) reliably induced chemical LTP (177 ± 12% one hour after LTP induction, n=14). Representative fEPSPs (single sweeps) were taken at the time points indicated in the graph. (B) Chemical LTP was long-lasting (137 ± 11% five hours after induction, n=5, subset of A). (A, B) Baseline: ten minutes prior to the application of the potentiation medium.

activation, but that a residual component is dependent on voltage-dependent calcium channels (Hanse and Gustafsson, 1994; Huber et al., 1995). Indeed, a complete block of chemical potentiation was obtained by a combination of AP5 and the L-type calcium channel blocker verapamil (102 ± 6% one hour after LTP induction, n=7, in two rats, Fig. 4.10B). AP5 (100µM) and verapamil (30µM) were present in the extracellular medium 20 minutes before, during and 40 minutes after the bath application of potentiation medium. After the wash-out of AP5 and verapamil, tetanic LTP could be induced normally (131 ± 5% one hour after tetanus, n=7, in two rats, Fig. 4.10B).

Standard electrical CA3-CA1 LTP induced by a high-frequency tetanus (100Hz) requires an increase in postsynaptic calcium (Lynch et al., 1983; Malenka et al., 1988) mediated by the activation of NMDA receptors (Collingridge et al., 1983; 1988). Interestingly, electrical LTP induced at very high frequencies (≥ 200Hz) recruits additionally voltage-dependent calcium channels (Grover and Teyler, 1990). My experiments have confirmed previous studies reporting that chemical LTP utilizes both routes of postsynaptic calcium entry, namely activation of NMDA receptors and voltage-dependent calcium channels (Hanse and Gustafsson, 1994; Huber et al., 1995).

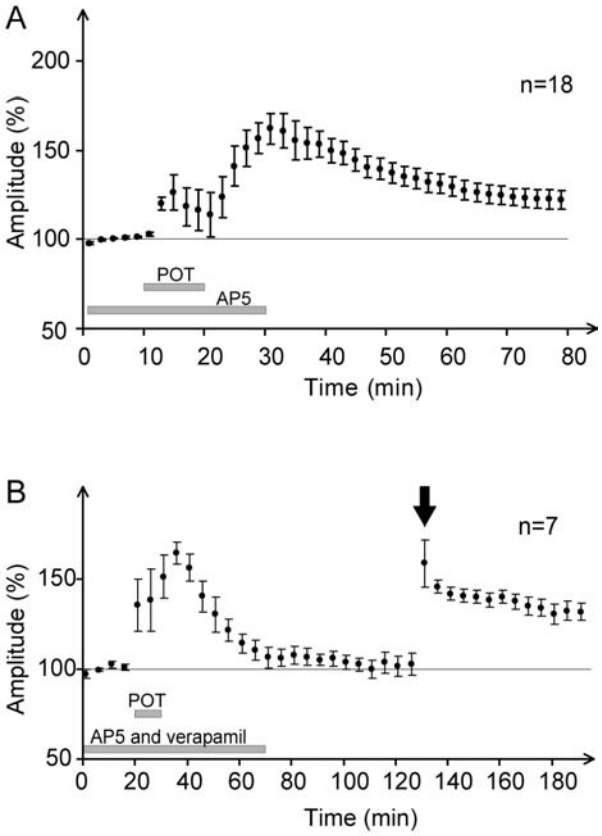


Figure 4.10 Pharmacological sensitivity of chemical LTP. (A) The NMDA-receptor antagonist AP5 (50µM) reduced chemical LTP (123 ± 5% one hour after LTP induction, n=18). Baseline: ten minutes prior to the application of the potentiation medium. (B) Chemical LTP is completely blocked by 100µM AP5 and 30µM of the L-type calcium channel blocker verapamil (102 ± 6% one hour after LTP induction, n=7). After the washout of AP5 and verapamil, tetanic LTP could be induced normally (131 ± 5% one hour after tetanus, n=7). Baseline: twenty minutes prior to the application of the potentiation medium. Arrow: tetanus (100 pulses, 100Hz).

Occlusion Experiments

Another strategy to assess homology between different ways of LTP induction is testing for occlusion. Occlusion experiments are frequently used to determine whether different forms of synaptic plasticity share common mechanisms (e.g. Cormier et al.,

1993; Kauer et al., 1988). If the respective mechanisms are saturated by one form of LTP induction, all capacity for further synaptic strengthening is 'used up'. Thus, applying another form of plasticity that acts through the same mechanisms, does not lead to an additional increase. This is called 'occlusion'. Consequently, if chemical LTP uses the same mechanisms as electrical LTP, prior chemical LTP induction should block subsequent electrical LTP on the same synapses. I performed this type of occlusion experiment to confirm the similarity of chemical and electrical LTP. Potentiation medium was bath applied and chemical LTP was monitored for one hour. Subsequently, a tetanus was applied to the same afferents. I observed that previous chemical LTP occluded later tetanic LTP (chemical LTP: 221 ± 25%, 50 minutes after application of potentiation medium, no tetanic LTP: 98 ± 8% one hour after tetanus, n=5, in two rats, Fig. 4.11A). Before the tetanus was delivered, a second baseline was recorded and the strength of test stimulation was reduced to evoke fEPSPs of the same amplitude as during the initial baseline. Reducing the stimulation strength ensures that the already potentiated synapses experience the same conditions for the second LTP induction as for the first (Frey et al., 1995) and this is the way occlusion experiments are usually performed.

Interestingly, if stimulation strength was not reduced, additional tetanic LTP could be induced on top of the chemical potentiation (Fig. 4.11B, chemical LTP: 201 ± 1%, one hour after application of potentiation medium; tetanic LTP: 131 ± 1%, one hour after tetanus, n=4, in two rats). The additional potentiation was due to the stronger cooperative effect of a bigger EPSP.

Occlusion experiments and the similar pharmacological sensitivity indicate that chemically-induced LTP and electrically-induced LTP share common mechanisms. Therefore, chemical LTP is a valid replacement of tetanus LTP to achieve widespread synaptic strengthening throughout the slice.

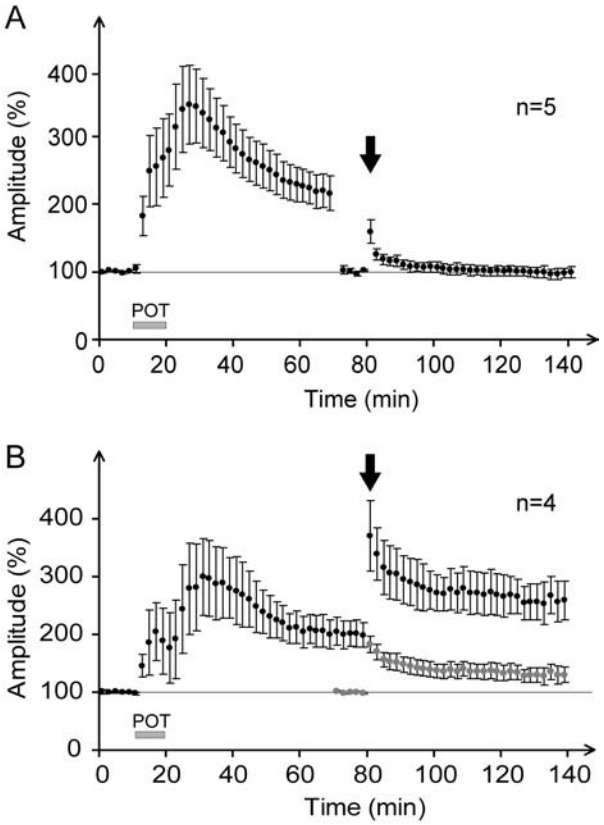


Figure 4.11 Occlusion Experiments. (A) Previous chemical LTP occluded later tetanic LTP (chemical LTP: 221 ± 25% 50 minutes after application of potentiation medium, no tetanic LTP: 98 ± 8% one hour after tetanus, n=5). At 70 minutes, the stimulation strength was reduced to evoke fEPSPs of the same amplitude as during the initial baseline. Baseline: ten minutes prior to the application of the potentiation medium. (B) If stimulation strength was not reduced, additional tetanic LTP could be induced on top of the chemical potentiation (chemical LTP: 201 ± 1% one hour after application of potentiation medium, baseline: ten minutes prior to the application of the potentiation medium). Grey data points represent the tetanic LTP normalized to a new baseline ten minutes prior to the tetanus (131 ± 1% one hour after tetanus, n=4). Arrow: tetanus (100 pulses, 100Hz).

4.3.2 Superfusion Experiments: Homeostasis upon widespread synaptic strengthening?

The purpose of these experiments was to find out whether LTP induction at a large proportion of synapses evokes homeostatic regulation. Accordingly, chemical potentiation was induced throughout the entire slice except for some synapses within a superfusion spot – the test synapses (Fig. 4.12, Fig. 4.13; Engert, 1997). Afterwards, the superfused and hence unpotentiated test synapses were probed for their capability to undergo LTP. A single electrical tetanus was used for this purpose as in the experimental series before. Probing the test synapses for LTP was the last and most important part of the whole experiment. To get there however, a sequence of experimental steps had to be taken (schematic representation and example of a single experiment, Fig 4.13 A).

Throughout the experiment, baseline stimulation was performed via a stimulating electrode in the CA3 region. Extracellular field potentials were recorded in stratum radiatum of CA1, on the surface of the slice because the superfusion solution penetrates only about 15 to 30µm deep into the tissue (as assessed by electrophysiological measurements, see Methods). The superfusion solution contained a higher calcium concentration (10mM, for explanation see Methods), which led to an increase in fEPSP amplitude once the superfusion was directed onto the slice close to the recording electrode. Subsequently, synaptic transmission outside the superfusion spot was blocked by replacing the normal extracellular medium with a solution containing a low calcium concentration (1.2mM) and 5µM cadmium (see Methods, Fig. 3.3). The decrease in signal amplitude revealed that under normal recording conditions, synapses from both inside and outside the superfusion spot contribute to the fEPSP. The initial blocking period was performed routinely to establish the optimal position of the stimulating electrode, the recording electrode and the superfusion spot. After changing the blocking solution back to normal ACSF, the signal recovered (Fig. 4.13). Subsequently, the potentiation medium was bath applied for ten minutes to induce chemical LTP throughout the whole slice except for those synapses within the superfusion spot. The overall signal amplitude of the compound fEPSP increased, due to potentiation of the outside-spot fraction of synapses (both inside-spot and outside-spot synapses contribute to the fEPSP). Chemical LTP was monitored for one hour and then blocking solution was washed in a second time. Field EPSP amplitude decreased again, because the outside-spot fraction of synapses was silenced. Thereby, I could exclusively monitor the inside-spot synapses. These are the test synapses, and to probe their capability to undergo LTP was the ultimate goal of this experiment. After recording a stable baseline, a tetanus was applied to the inside-spot synapses via the stimulating electrode. Clear post-tetanic potentiation was observed indicating normal synaptic functioning, but signal amplitudes rapidly decayed back to baseline levels.

Control experiments were performed according to the same protocol and the same recording conditions but without chemical potentiation (schematic representation and example of a single experiment Fig. 4.13B). In this case, inside-spot synapses showed robust LTP upon tetanic stimulation.

The summary analysis in Figure 4.13C focuses on this last part of the whole experiment and

compares the potentiability of inside-spot synapses in slices with or without previous chemical LTP. Following widespread synaptic strengthening, later LTP induction at hitherto unpotentiated synapses was not possible anymore (test experiments, 98 ± 3%, one hour after tetanus, n=6, in six rats, Fig. 4.15C). When previous large-scale potentiation had not taken place, normal LTP was expressed (control experiments, 136 ± 11%, one hour after tetanus, n=5, in five rats, $p<0.05$, Fig. 4.15C). This data set supported the view, that widespread synaptic strengthening can indeed lead to homeostatic shut-down of LTP.

However, a caveat appeared on the scene. Comparison of the signal amplitudes during the first and second blocking periods revealed an unexpected increase of the inside-spot fEPSP after chemical LTP induction outside (fEPSPs during the second blocking period relative to those during the first: 145 ± 8%, n=8, in eight rats, see Fig. 4.13A for example experiment and Fig. 4.14A for summary). Different reasons might account for this undesired potentiation of inside-spot synapses; but whatever the reason was, the unexpected and undesired inside-spot potentiation made an unambiguous interpretation of the

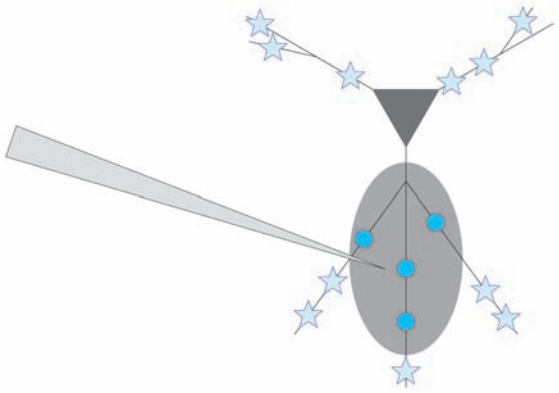
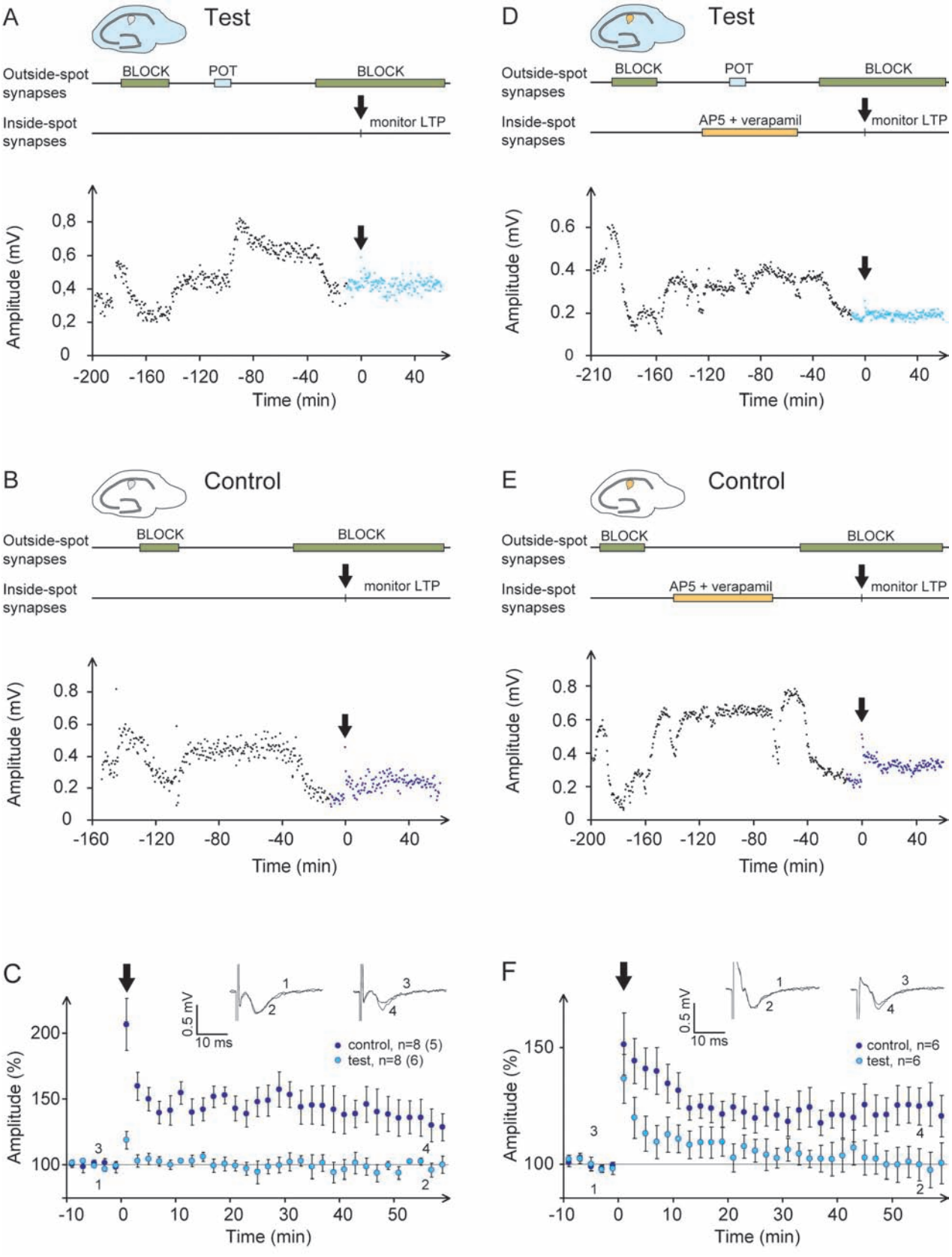


Figure 4.12 Experimental design of the superfusion experiments. The synapses outside the superfusion spot are potentiated by chemical LTP (stars). The test synapses inside the superfusion spot (blue dots) are spared from potentiation medium and will subsequently be probed for LTP.

superfusion results difficult. The shut-down of LTP might have been the consequence of homosynaptic occlusion (which has been shown before, e.g. Frey et al., 1995) rather than heterosynaptic homeostasis.

Therefore, the entire superfusion experimental paradigm was repeated under circumstances where intrusion of chemical LTP into the spot was pharmacologically prevented (schematic representation and example of a single experiment Fig. 4.13D). The regime of

Figure 4.13 Superfusion experiments. (A, B) Experimental design and representative example of a test experiment (A) and a control experiment (B). Outside-spot synapses: synapses outside the superfusion spot, inside-spot synapses: synapses within the superfusion spot. BLOCK: bath application of blocking medium, POT: bath application of potentiation medium. Blocking and potentiation medium have access to the outside-spot synapses, but not to the inside-spot synapses. A tetanus was applied at time point zero. Baseline: ten minutes prior to the tetanus, arrow: tetanus (100 pulses, 100Hz). Coloured data points contribute to the summary graph in C. (C) No LTP could be induced after widespread synaptic strengthening (test experiments: 98 ± 3%, one hour after tetanus, n=6). When previous large-scale potentiation had not taken place, normal LTP was expressed (control experiments, 136 ± 11%, one hour after tetanus, n=5). The difference between control and test experiments was significant ($p<0.05$). Not all of initially eight test experiments and eight control experiments lasted until one hour after tetanus. Representative fEPSPs averaged from five consecutive stimuli were taken before and after tetanus at the time points specified in the graph.
(D-F) Same as A-C, but the NMDA-receptor antagonist AP-5 (100µM) and the L-type calcium channel blocker verapamil (30µM) were present in the superfusion solution 20 minutes before, during and 40 minutes after the bath application of potentiation medium. This second series of superfusion experiments with pharmacological protection of the superfusion spot confirmed the observation of the first series: no LTP following widespread synaptic strengthening (test experiments: 100 ± 7%, one hour after tetanus, n=6) and normal LTP in control experiments (124 ± 8%, one hour after tetanus, n=6). The difference between test and control experiments was significant ($p<0.05$).


A
Test
Outside-spot synapses
BLOCK
POT
BLOCK
Inside-spot synapses
monitor LTP
Amplitude (mV)
Time (min)
B
Control
Outside-spot synapses
BLOCK
BLOCK
Inside-spot synapses
monitor LTP
Amplitude (mV)
Time (min)
C
0.5 mV
10 ms
control, n=8 (5)
test, n=8 (6)
Amplitude (%)
Time (min)
D
Test
Outside-spot synapses
BLOCK
POT
BLOCK
Inside-spot synapses
AP5 + verapamil
monitor LTP
Amplitude (mV)
Time (min)
E
Control
Outside-spot synapses
BLOCK
BLOCK
Inside-spot synapses
AP5 + verapamil
monitor LTP
Amplitude (mV)
Time (min)
F
0.5 mV
10 ms
control, n=6
test, n=6
Amplitude (%)
Time (min)

the superfusion experiment was changed so that the NMDA-receptor antagonist AP5 and the L-type calcium channel blocker verapamil (30µM) were present in the superfusion 20 min before, during and 40 min after bath application of the potentiation medium. Previous pilot experiments had demonstrated that this concentration and application time of AP5 and verapamil was sufficient to block chemical LTP in slices (for detailed explanation see above and Fig. 4.10). Indeed, adding AP5 and verapamil to the superfusion solution during bath application of the potentiation medium, prevented the potentiation of the synapses inside the superfusion spot (fEPSPs during the second blocking period relative to those during the first: 87 ± 10%, n=6, in six rats, Fig. 4.14B). A double-barreled superfusion system allowed the fast exchange of superfusion solutions without changing the position of the spot (Veselovsky et al. 1996). Using this double-barreled superfusion device (Fig. 3.5), normal superfusion solution without AP5 or verapamil was reintroduced, and test synapses inside the spot were then probed for LTP. Under these recording conditions, the result was the same as for the first superfusion series.

The test synapses in the superfusion spot were not able to undergo LTP after the outside-spot synapses had been potentiated chemically (100 ± 7%, one hour after tetanus, n=6, in six rats). Yet, under control conditions, i.e. without potentiation of the outside-spot synapses, normal LTP occurred (124 ± 8%, one hour after tetanus, n=6, in six rats). This difference was statistically significant ($p<0.05$, one hour after tetanus, Fig. 4.13F).

Thus, the second series of superfusion experiments under stricter pharmacological control yielded the same results as the first series and thus confirmed the observation of homeostatic shut-down after widespread synaptic strengthening.

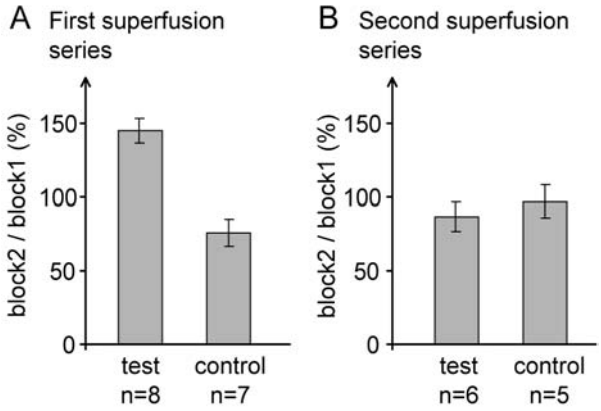


Figure 4.14 Field EPSP amplitudes of the inside-spot synapses. (A) Potentiation of inside-spot fEPSPs after chemical potentiation outside. Comparison of the signal amplitudes during the first and second blocking periods revealed an unexpected increase of the inside-spot fEPSP after chemical LTP induction outside (fEPSPs during the second blocking period relative to those during the first: 145 ± 8%, n=8). In control experiments, the ratio of fEPSPs during the second period relative to those during the first was 76 ± 8%, n=7. One control experiment could not be used for this analysis. This difference was significant ($p<0.05$). (B) Inside-spot potentiation was blocked by AP5 and verapamil. The NMDA-receptor antagonist AP5 and the L-type calcium channel blocker verapamil (30µM) in the superfusion prevented potentiation of inside-spot synapses (fEPSPs during the second blocking period relative to those during the first: 87 ± 10%, n=6). There was no significant difference to control experiments (fEPSPs during the second blocking period relative to those during the first: 97 ± 11%, n=5.) One control experiment could not be used for this analysis.

Amount of chemical potentiation in the superfusion experiments

Chemical potentiation was used as a means to obtain synaptic strengthening in a large proportion of synapses. Pilot experiments had revealed that induction of chemical LTP was highly reliable with a success rate of more

than 90%. Reliable induction of chemical LTP was very important, because any homeostatic effect could only be expected after successful LTP induction at a large number of synapses throughout the slice.

In an ideal experiment, a second recording electrode would be placed outside the superfusion spot to monitor the amount of chemical potentiation; but due to the technical complexity of the experimental design, this second electrode was not introduced. However, the recording electrode in the superfusion spot could also be used to address that question. When synaptic transmission outside the superfusion spot was not blocked (i.e. normal ACSF in the bath), the recording electrode picked up synaptic activity from both inside and outside the superfusion spot. Chemical potentiation outside the superfusion spot was thus reflected in an increase of the compound inside-spot/outside-spot fEPSP.

The summary graph in Figure 4.15A focuses on the time period of chemical LTP induction in the first superfusion series and shows that chemical potentiation was expressed consistently (135 ± 8%, one hour after wash-out of potentiation medium, n=8, in eight rats). Because induction and monitoring of chemical LTP took place in the absence of blocking medium, both inside-spot and outside-spot synapses contributed to the recorded fEPSP. The potentiation of the outside-spot synapses lead to the overall increase of the fEPSP, thus giving evidence that chemical LTP induction was successful.

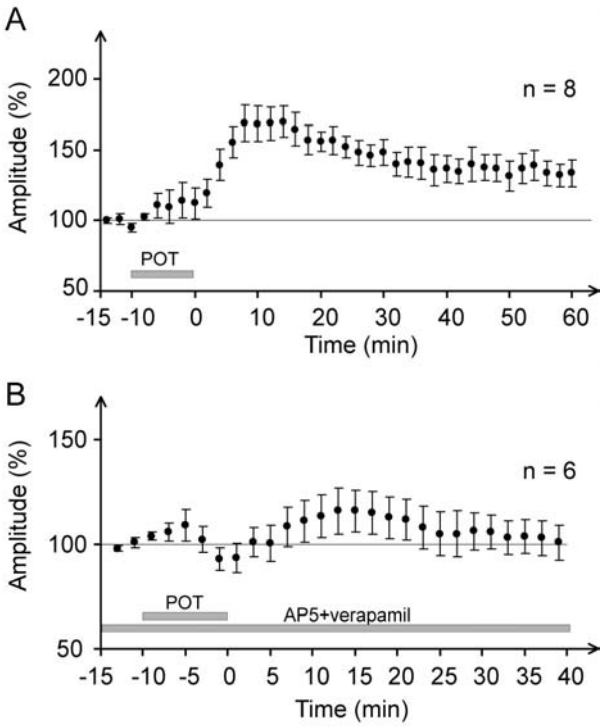


Figure 4.15 Amount of chemical potentiation in the superfusion experiments. The summary graphs focuse on the time period of chemical LTP induction in (A) the first superfusion series and (B) the second superfusion series. Because induction and monitoring of chemical LTP took place in the absence of blocking medium, both inside-spot and outside-spot synapses contributed to the recorded fEPSP.

In the first superfusion series, some of the synapses at the border of the superfusion spot were potentiated as well. Still, some fraction of the fEPSP remained unpotentiated, namely those synapses within the superfusion spot that were protected from chemical potentiation. Therefore, the absolute amount of chemical potentiation cannot be measured accurately by a recording electrode in the superfusion spot. Presumably, the 'true' amount of potentiation within the slice was higher than the value measured by the inside-spot recording electrode. This explains why the amount of chemical LTP in the first superfusion series appeared to be lower than that obtained in normal slice experiments (135 ± 8% as compared to 177 ± 12%). Only

a second recording electrode outside the superfusion spot could have determined the precise amount of potentiation, but it was not necessary for the interpretation of the results to obtain this value.

The same analysis was also performed for the second superfusion series. Here, AP5 and verapamil were present in the superfusion solution during the time of chemical LTP induction and prevented any undesired chemical potentiation of inside-spot synapses. In this case, the compound fEPSP was not different to baseline forty minutes after wash-out of potentiation medium (103 ± 8%, n=6, in six rats, Fig. 4.15B). Thus, most if not all synapses contributing to the field signal remained unpotentiated. This might be an indication that the 'mixing zone', where superfusion and bath solution mixed, was not so small after all. Reassuringly, the typical 'hump' fifteen minutes after application of the potentiation medium was still observed, indicating that the medium must have been effective. Given the reliability of chemical LTP, confirmed by the pilot experiments and the first superfusion series, it could be safely assumed that chemical LTP was successfully induced at outside-spot synapses in this second superfusion series as well.

4.3.3 Number of synapses in the superfusion spot

My data show that shut-down of LTP only occurred after widespread synaptic strengthening. I wanted to know what proportion of a neuron's synapses has remained unpotentiated. To this end, I used morphological data to estimate the number of synapses within the superfusion spot.

During all superfusion experiments, the superfusion spot was visually controlled. Additionally, in the second superfusion series, photographs of the slice and the superfusion spot were taken for a random subset of experiments (10 experiments altogether: 5 potentiation experiments, 5 control experiments). The dimensions of the superfusion spot were measured with image analysis software (ImageJ, public domain). The spot had a drop-like shape; in its longest extensions, the length was on average 510 ± 25µm and the width 268 ± 14µm (Fig. 4.16). The average surface area of the superfusion spot was 93350 ± 8950µm^2.

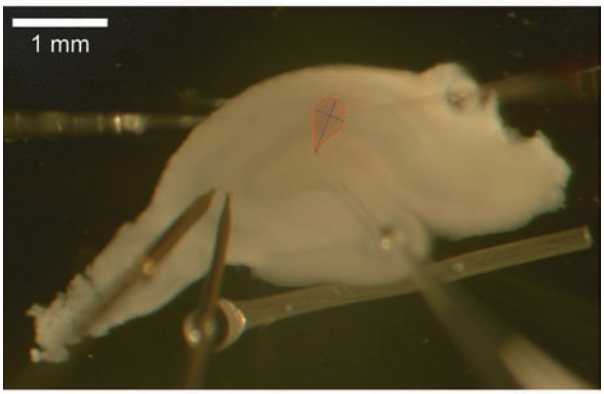


Figure 4.16 Photograph a hippocampal slice and a representative superfusion spot. The spots had a drop-like shape. In its longest extension, the length was on average 500 ± 25µm and the width 268 ± 25µm; the average surface area was 93359 ± 8950µm^2 (n=10).

Using electrophysiological recordings, I estimated that the superfusion solution penetrated approximately 30 µm deep into the slice (see methods). Hence the volume of the average superfusion spot was roughly 2,800,500 ± 268,500 µm^3 (= 30µm * (93,350 ± 8950µm^2)).

The apical dendritic tree of a typical CA1 neuron has a maximal extension of 500µm x 490µm x 280µm (Megías et al., 2001, see Fig. 4.17), i.e. the cuboid that represents the maximal extension of the apical dendrites has a volume of 68,600,000µm^3.

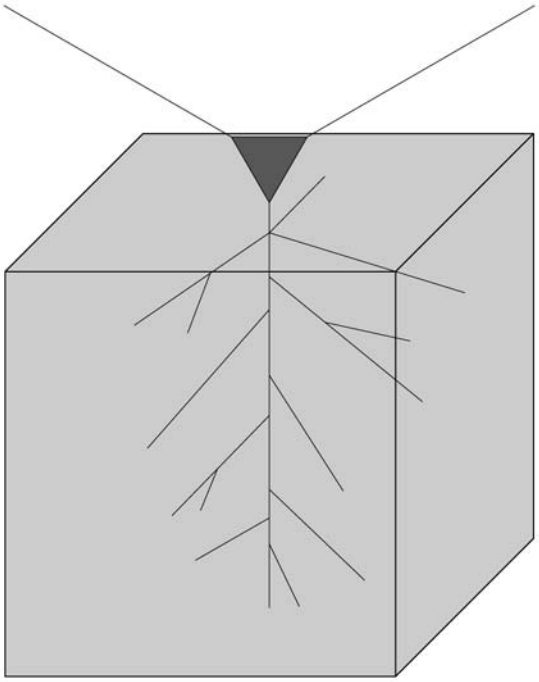


Figure 4.17 The apical tree of CA1 neurons. The grey box represents the maximal extension of the apical dendrites which is on average 500µm x 490µm x 280µm (Megias et al., 2001).

I estimated that the superfusion spot occupied about 4% percent of this apical extension volume. A rat hippocampal CA1 neuron bears approximately 32350 synapses, 19730 of those are on the apical dendrites (Megías et al., 2001). Assuming that all synapses are equally distributed within the apical cuboid, around 790 (= 4% of 19730) synapses would lie within the superfusion spot, in other words 2.4% of the total number of synapses on a CA1 neuron.

5 Discussion

I wanted to find out whether neurons can regulate the 'potentiability' of their synapses to avoid 'runaway' Hebbian potentiation. To this end, I performed experiments in rat hippocampal slices using classical NMDA-receptor dependent CA3-CA1 LTP. I have demonstrated that neurons can sustain substantial amounts of synaptic strengthening. However, when a large number of synapses have undergone LTP, further potentiation at other synaptic sites is no longer possible.

There are many cell-biological reasons for neurons to enlist homeostatic processes controlling, for example, ion concentrations, osmotic conditions, oxygen consumption, energy metabolism and such. In addition, neurons not only have to monitor their cell biological parameters, but for the proper functioning of neural circuits it is of paramount importance that neural activity is carefully regulated.

5.1 Extracellular Experiments

My first set of experiments were designed to examine whether potentiation in one Schaffer collateral pathway of the hippocampus might prevent or diminish further potentiation off neighbouring synapses from a different pathway (Fig. 5.1A). If some homeostatic mechanism were to limit the total amount of potentiation within a population of synapses, prior LTP induction might block or at least reduce the LTP in another subset of synapses. Yet, both the strong and the weak form of the initially formulated homeostatic hypothesis were refuted: Potentiating one pathway neither blocked nor reduced potentiation on a second, independent input onto the same neurons.

However, LTP in the first pathway was not particularly high and decayed rather rapidly. Probably, only an early, protein-synthesis independent form of LTP had been induced which may not have caused the necessity of homeostatic intervention. In view of that, I modified my homeostatic hypothesis. The initial experiments demonstrated that normal,

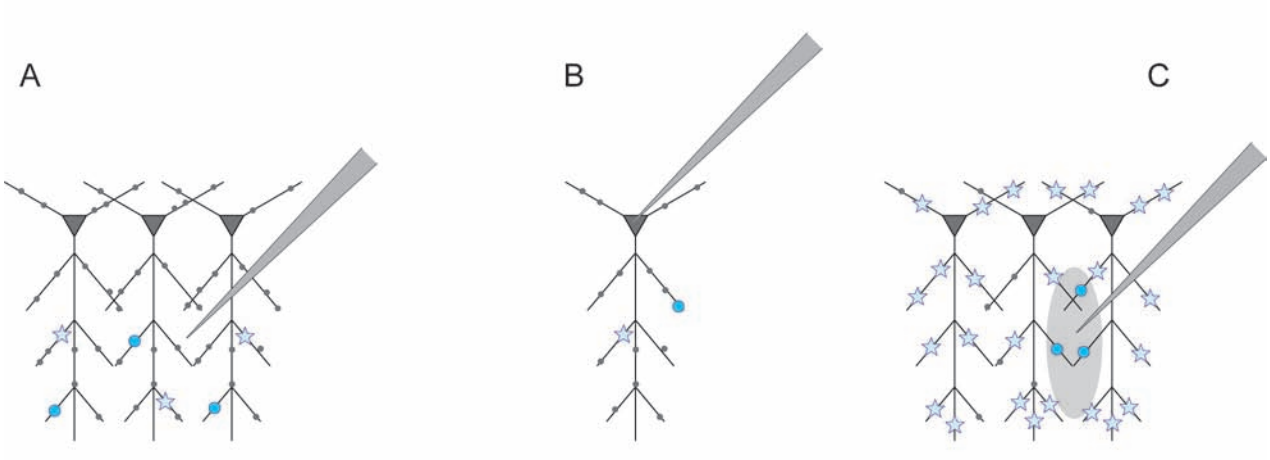


Figure 5.1 Schematic representation of the different recording conditions. (A) Extracellular experiments. (B) Intracellular experiments. (C) Superfusion experiments. Stars: synapses potentiated by either (A, B) saturating LTP induction or (C) chemical LTP. Blue dots: test synapses. Grey dots: non-activated synapses.

transient LTP did not interfere with further LTP induction at other synaptic sites, but I speculated that saturated and long-lasting LTP might well do. Accordingly, I adapted the experimental paradigm to induce saturated LTP in the first pathway, which was long-lasting (six hours) and extended into the protein-synthesis dependent time range.

Even under the conditions of saturated LTP on one pathway, potentiation of an independent input onto the same neurons was neither blocked nor reduced. Also the possibility that early LTP would not show any homeostatic effect, but only late LTP was not upheld.

At first glance, the results of the extracellular recordings seem to argue against homeostatic regulation of synaptic efficacy, but they might not be fully conclusive. There is one important caveat implicit in the extracellular approach I had used. In extracellular recordings, the populations of CA1 neurons stimulated by the two pathways might not fully overlap. Most certainly, a majority of neurons would receive input from both pathways – these are the neurons of interest – but I cannot rule out the possibility that neurons receiving input solely from one of the two pathways are included (Fig. 5.2).

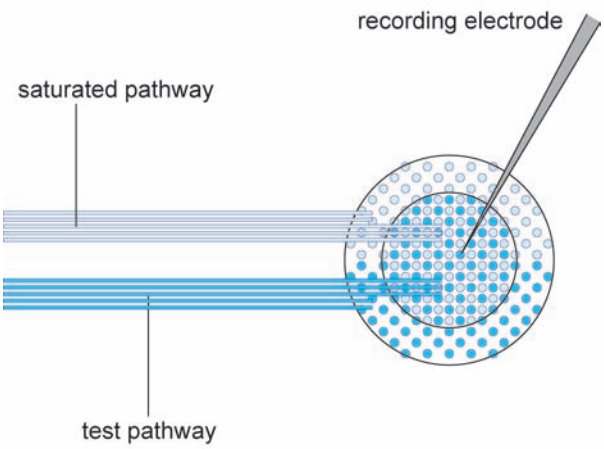


Figure 5.2 The populations of CA1 neurons stimulated by the two pathways might not fully overlap. A majority of neurons will receive input from both pathways (inner concentric circle), but there might also be neurons that receive input from only one pathway (outer concentric circle). Light blue dots: synapses activated via the saturated pathway, middle blue dots: synapses activated via the test pathway.

Specifically, those neurons receiving input only from the test pathway would be expected to exhibit normal LTP in response to the test tetanus, as they have never been subject to a saturating stimulus. The contribution of these neurons to the recorded field potential might mask any homeostatic effect, misleadingly interpreted as its non-existence. I decided to address this issue with intracellular recordings from single CA1 neurons.

5.2 Intracellular Experiments

With regard to the stimulation, the experimental paradigm was very similar to that used for the extracellular saturation experiments. The single cell approach could provide a conclusive answer as to whether the applied stimuli entail a homeostatic response of the neuron (Fig. 5.1B). Control experiments, showed that LTP induction was still possible

after 90 minutes recording time and therefore established that the single cell approach was suitable (Fig. 4.5).

According to the homeostatic hypothesis, the saturation experiments should exhibit a complete lack of LTP in the test pathway or at least a reduction as compared to controls. But much to my dismay, I observed that despite apparently saturating LTP induction on the first pathway, CA1 neurons still exhibited additional potentiation in the test pathway in response to a tetanus. Surprisingly, LTP in the test pathways even appeared slightly higher than that of the control experiments, although the difference was not statistically significant. In my view, this does not reflect a supposed facilitating effect of LTP saturation on the first pathway. One has to consider that neurons recorded in the saturation experiments were positively selected for strong potentiation by the saturation criteria. This kind of positive selection however did not take place for the control experiments, which explains why potentiation in the control pathways (no pre-selection) was slightly lower than potentiation in the test pathways (with pre-selection). Furthermore, most control experiments were performed early in the series when technical skills were less developed than towards the end.

A limitation of the intracellular approach was that I could not monitor the magnitude and decay of this potentiation over long periods, so one might argue that homeostasis only occurs over longer time frames (of a couple of hours) and that I would not be able to observe it using this approach. While I cannot completely exclude that, I find it reasonable to expect some negative correlation between the magnitude of the LTP in the saturated pathway and that of the LTP in the test pathway even early on. The data however did not show any negative correlation whatsoever; if anything, a weak positive correlation was observed (Fig. 4.8). A similar positive correlation was also observed for the extracellular saturation experiments (Fig. 4.3). This means that neurons which exhibited high potentiation in the saturated pathway also showed strong potentiation in the test pathway. Thus, some slices seemed to have a higher intrinsic potentiability than others. One reason might be that these slices have sustained the preparation procedure better than others, resulting in a better health of the neurons, which enabled them to follow more repetitions of tetanus trains. Another reason could be the history of the respective synapses. Synapses that were 'pre-potentiated' before they arrived *in vitro*, either by learning experiences of the animal or high overall neuronal activity in the slice, might have less capacity for further potentiation (Abraham and Tate, 1997).

Interestingly, it never occurred that an intracellularly recorded neuron received input from only one stimulation pathway. This observation shed new light on the concern that the population of neurons contributing to the extracellular field potential might have been heterogeneous. Of course, it still cannot be ruled out completely that a single neurons of that population only received input from the test pathway, but it does not seem very likely. Even more so, because the stimulation strength in the extracellular recordings was about twice as high as for the intracellular recordings, which means that more afferents were recruited for a given stimulus. Activating more afferents increases the likelihood that a neuron receives input from both pathways. For these reasons, the theoretical concern of a heterogeneous extracellular recording population can be neglected, which validates

the extracellular data in retrospect. The apparent absence of a homeostatic regulation of potentiability in both extracellular and intracellular recording conditions, suggests that – as a matter of fact – it did not occur with the stimulation pattern applied.

5.3 Self-normalization through spike-timing dependent plasticity?

Within certain limits, neurons may allow their synaptic population to regulate their efficacy relatively independently. Spike-timing dependent plasticity provides an immediate mechanism for self-normalization of synaptic weights and the output firing rate of a network (Kempter et al., 2001, Song et al., 2000; Senn et al., 2001; Bi and Poo, 2001). In cell culture, a critical window for the induction of LTP/LTD has been characterized by systematically varying the spike timing, defined as the time interval between the onset of the presynaptic and the postsynaptic spike (Fig. 5.3; Bi and Poo, 1998).

The time window for modification is temporally asymmetric: Postsynaptic spiking after presynaptic activation (positive intervals) results in LTP, whereas positive spiking before presynaptic activation (negative intervals) results in LTD. Interestingly, the time window for depression is longer than that for potentiation. This feature of STDP may help to keep synaptic weight changes in balance.

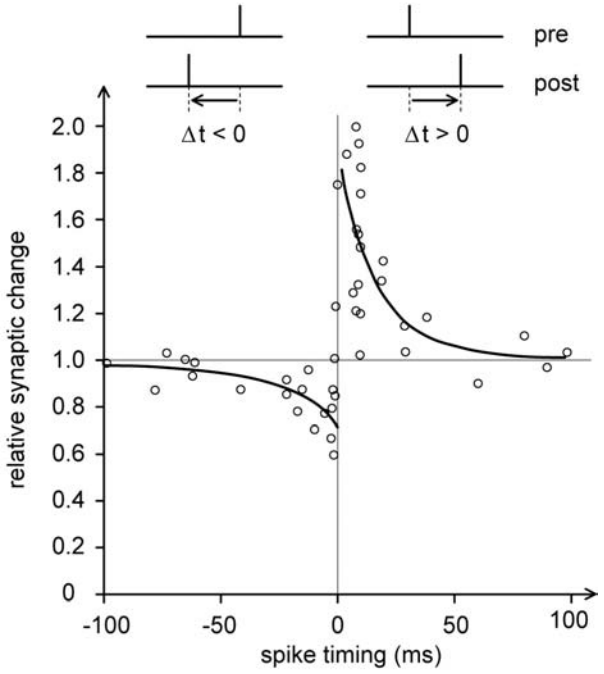


Figure 5.3 Spike-timing dependent plasticity: critical window for synaptic modifications. Each data point represents the relative change in the amplitude of EPSC after repetitive application of pre- and postsynaptic spiking pairs with a fixed spike timing Δt. LTP and LTD windows are each fitted with an exponential function. Adapted from Bi and Poo, 2001.

In a natural setting, a synapse continuously experiences pre- and postsynaptic spike trains that often contain multiple spikes within tens of milliseconds. The final outcome of synaptic modification may then be viewed as integration of several spike pairs, each of which interacts to produce synaptic modifications according to the spike timing rule (Bi and Wang, 2002). When excitatory synapses become potentiated, the firing rate in a neuronal network will go up. A higher number of action potentials increase the probability that pre- and postsynaptic spikes are paired within a time window relevant for STDP. As the time window for depression is longer than that for potentiation, spike combinations leading to LTD might outnumber the spike pairs leading to LTP, which would lead to a net decrease of synaptic strength. In this way,

spike timing dependent plasticity may act to normalize synaptic weights. Hence, under many circumstances, changes in synaptic strength might be sufficiently equilibrated by the ongoing neuronal activity. Indeed, in the developing Xenopus retinotectal system, LTP was quickly reversed either by subsequent spontaneous activity or by random visual inputs (Zhou et al., 2003). Although such a reversal of synaptic modifications might only occur in vivo and not in vitro because of lacking spontaneous activity, it may explain why I did not observe homeostatic regulation of LTP in the extracellular and intracellular experiments. When the amount of synaptic potentiation remains below a certain threshold, neurons may simply rely on the ongoing normalization through neuronal activity instead of shutting down LTP.

While it was tempting to abandon my search for homeostatic regulation at this point, I also wondered whether the 'saturation' of one pathway is quite the same as the induction of LTP at a high proportion of a neuron's afferents. The operational definition of saturation was that strong potentiation should be observed on the 'saturating' pathway that did not increase with further tetanizing trains. This definition ensured that every synapse activated by the particular stimulation electrode was maximally potentiated with the specific stimulus protocol applied. (A stronger tetanus though might have been able to further strengthen the synapse.)

Although the *individual* synapses were highly potentiated, it may be that the *total* number of potentiated synapses on the postsynaptic neuron remained within a working range after tetanization of a single pathway and thus below some threshold required to trigger homeostasis.

5.4 Superfusion Experiments

Accordingly, in a final series of experiments, I turned to an alternative protocol for inducing LTP, which, I reasoned, should result in a much larger proportion of synapses on individual neurons being potentiated (Fig. 5.1C). This involved chemical potentiation. Under these circumstances, I found that indeed, later electrical potentiation of LTP at hitherto unpotentiated synapses was no longer possible. In control experiments, normal LTP was expressed. These data indicate that widespread synaptic strengthening can indeed lead to homeostatic shut-down of LTP (Fig. 4.13).

It was important though to confirm that chemical potentiation is equivalent to the conventional electrical potentiation. Otherwise, the observed shut-down might just be regarded as an artifact of the chemical potentiation. The analogy in pharmacological sensitivity established that chemical and electrical LTP have similar properties and share common signaling pathways (see Fig. 4.10 and also Aniksztein, 1991; Song et al., 2001).

Occlusion of electrical potentiation by prior chemical potentiation was another indication that both forms of LTP engage the same cellular mechanisms. Complete occlusion was observed when the stimulation strength of the test pulse was reduced after chemical LTP so that during the second baseline before the electrical tetanus, the fEPSPs had the same amplitude as during the initial baseline (Fig. 4.11A). If the stimulation strength was not reduced, only incomplete occlusion was observed (Fig. 4.11B). How can this be explained? In comparison to the normal baseline, the bigger fEPSP has a greater

cooperative effect. Thus, the same synapses can be further potentiated, probably by additional calcium influx through voltage-dependent calcium channels that become activated when the cooperativity effect is large enough. This cooperativity effect is not particular to chemical LTP, but is also of relevance when the occlusion of electrical LTP by previous electrical potentiation is tested (Frey et al., 1995). Regarding my attempt to demonstrate the homology of chemical and electrical potentiation it was crucial that under the same baseline conditions, no electrical LTP on top of chemical LTP was observed.

In summary, pharmacology as well as the occlusion experiments indicated that chemical and electrical LTP can be used exchangeably for my purposes. Hence, I am convinced that it is the proportion of synapses potentiated rather than the way of LTP induction that triggers homeostatic shut-down.

However, no sooner had my search for the hitherto cryptic homeostatic regulation of LTP proved successful than a caveat appeared on the scene. Post-hoc data analysis revealed that inside-spot synapses also became potentiated after chemical LTP induction outside, although exactly this should have been prevented by the local superfusion technique (Fig. 4.14). Different reasons might account for this undesired potentiation of inside-spot synapses:

1. Engert and Bonhoeffer (1997) have shown that synapse specificity of LTP breaks down at short distances (<70μm). Chemically induced LTP at synapses outside the spot might have spread to neighboring synapses within the spot. This spread of LTP might have been the reason for inside-spot synapses to become potentiated, although they had not been in direct contact with the potentiation medium.

2. Another potential explanation might be the general increase in neuronal activity caused by the potentiation medium. Thereby, it became more likely that a presynaptic action potential was paired with postsynaptic depolarization in the appropriate time window for synaptic strengthening to occur. Spike-timing dependent plasticity might have happened at inside- and outside-spot synapses alike leading to the observed potentiation.

3. Finally and most likely, diffusion of potentiation medium into the spot might have been the cause of inside-spot potentiation. Most probably, the borders of the superfusion spot in acute hippocampal slices were not as sharp as reported for cultured tissue (Veselovsky et al., 1996) because the more uneven surface of the acute slice preparation might have interfered with laminar flow of the superfusion solution. At the borders of the superfusion spot, the superfusion solution might have mixed with the respective bath solution by diffusion. One has to take into account that the superfusion solution contained high calcium, whereas the blocking medium contained low cadmium. Consequently, a concentration effect might have taken place in this 'mixing zone' of unknown dimensions: low concentrations of potentiation medium might have been sufficient to induce potentiation, but low concentrations of blocking medium presumably did not suffice to block synaptic transmission. Accordingly, synapses in this area would have been potentiated even by a diluted potentiation medium. However, the very same synapses would not have been blocked later on, because dilution had rendered the blocking medium ineffective in the mixing zone. The observed increase from the first to the second blocking period

would be due to the potentiated synapses of the mixing zone contributing to the fEPSP recorded under slice blockade.

Yet, whatever the underlying cause, the unexpected inside-spot potentiation made a clear-cut interpretation of the superfusion results difficult. The shut-down of LTP might be the consequence of homosynaptic occlusion rather than heterosynaptic homeostasis. I therefore repeated the entire superfusion experiment under circumstances where intrusion of chemical LTP into the spot was pharmacologically prevented. This second series of superfusion experiments under strict pharmacological control confirmed the observation of homeostatic shut-down after widespread synaptic strengthening.

My data show that shut-down of LTP only occured after a relatively large population of synapses had been potentiated. Conventional electrical tetanic potentiation affected too few synapses to invoke a homeostatic mechanism, whereas widespread synaptic strengthening by chemical means resulted in a homeostatic shut-down of synaptic potentiation. Probably, there is some threshold of potentiation that has to be reached, before homeostatic mechanisms are engaged. So what proportion of synapses needs to be potentiated to trigger homeostatic shut-down of LTP?

5.5 Threshold for homeostatic shut-down

The experiment described above was not geared to determine the number of potentiated synapses necessary for shut-down of LTP and therefore, the question is not answered as yet. However, estimating the number of synapses activated by chemical or electrical potentiation can at least help to define an upper and a lower boundary between which the homeostatic threshold resides.

I estimated the number of synapses within the superfusion spot, which in turn gives a hint as to the proportion of synapses outside the spot that were subject to chemical potentiation. The size of the superfusion spot in my experiments ($93350 \pm 8950 \mu m^2$) was larger by an order of magnitude than in previous studies (Engert and Bonhoeffer, 1997, 1999; Polnau, 2003). There are two main differences between these studies and my work: the slice preparation and the recording technique.

The experiments of Engert and Bonhoeffer (1997) and Polnau (2003) were performed in organotypic hippocampal slice cultures whereas I used acute hippocampal slices. The slice cultures were only about 50 μm thick and the cell density was reduced to 2-3 cells per layer (Polnau, 2003). In contrast, the acute slices I used were thicker (350 μm) and the tissue was denser. Therefore, the superfusion solution had less access to the neurons than in the organotypical slice cultures.

The second important difference was the recording technique: I performed extracellular field recordings; Engert and Bonhoeffer (1997) and Polnau (2003) used intracellular recordings. The intracellular technique is more sensitive than the extracellular, because the recorded voltage changes are bigger. Consequently, a few active synapses in a small superfusion spot can be detected by an intracellular recording electrode but not by an extracellular one. For both of these reasons, namely less access of the superfusion solution to the neurons and a lower sensitivity of the recording technique, my superfusion

spot had to be considerably larger in order to obtain a decent signal-to-noise-ratio during the blocking phases of the superfusion experiment.

The volume of the average superfusion spot was roughly 2,800,500 ± 268,500 μm^3. Morphological studies using serial electron microscopy can give an accurate account of synaptic density, i.e. the absolute number of synapses within a certain volume of hippocampal tissue (e.g. Sorra and Harris, 1998). Sorra and Harris determined the 'mean-adjusted synaptic density' in area CA1 of adult rats to be around 350 ± 70 synapses/100μm^3 tissue. Accordingly, on the order of 9.8 ± 0.19 million synapses were to be found in the superfusion spots of my experiments.

To assess the homeostatic threshold, one needs to know how many synapses of an individual neuron are within the superfused volume. In relation to the total number of synapses on that neuron, the relation of non-potentiated to potentiated synapses could be calculated. This provides an upper estimate of the proportion of synapses that need to be potentiated to trigger homeostatic shut-down. Thus, the question for the superfusion experiments was: How many synapses of a CA1 neuron are within the superfusion spot and how many are outside? An experimental approach to answer that question would involve imaging techniques such as confocal and two-photon microscopy. One would image a fluorescently labeled CA1 neuron and the corresponding superfusion spot; create a three-dimensional reconstruction and overlay the spot onto the neuron. Subsequently, one could count the number of spines inside and outside the superfusion spot, corresponding to the number of excitatory synapses. The imaging approach probably would yield the most precise answer to the above question, but it is very laborious and beyond the scope of this study. Alternatively, I estimated the proportion of synapses inside and outside the superfusion spot. My 'educated guess' was based on my own measurements of the spot size and volume and detailed morphological studies of rat CA1 pyramidal neurons published in the last few years (e.g. Pyapali et al., 1998; Megías et al., 2001; Scorcioni et al, 2004).

Briefly: The apical dendritic tree of a typical CA1 neuron has an extension of 500µm x 490µm x 280µm (Megías et al., 2001, see Results), and I estimated that the superfusion spot occupied about 4% percent of this apical extension volume. A rat hippocampal CA1 neuron bears approximately 32350 synapses, of which 19730 are on the apical dendrites (Megías et al., 2001). Assuming that all synapses are equally distributed within the apical cuboid, around 790 (= 4% of 19730) synapses would lie within the superfusion spot, in other words 2.4% of the total number of synapses on a CA1 neuron. The critical step in this reasoning is the assumption of equal distribution of the synapses. The synapses of any individual neuron are of course not equally distributed, because every synapse is located on a dendrite and the dendritic tree has a defined shape. However, in the superfusion experiments I did not record individual neurons, but extracellular field potentials from a population of neurons. The dendritic trees and the synapses of these neurons, however, are equally distributed within a certain hippocampal volume. Therefore, the assumption of equal distribution seems to be justified; the only 'trick' is to calculate with the number of synapses of a single neuron, as the purpose of the whole calculation was to get an estimate of the proportion of potentiated synapses on individual neurons.

It is important to keep in mind that this was only a rough calculation, and probably an underestimate of the number of synapses in the superfusion spot (e.g. not all dendritic branches expand in the assumed maximal extensions of the dendritic tree). Therefore, assuming about 2-10% of the synapses of a single neuron were protected from the potentiation medium by superfusion, an upper estimate of the proportion of synapses that need to be potentiated to trigger homeostatic shut-down, may be as high as 90-98%, although I suspect it is much lower.

The extracellular and intracellular series show that conventional tetanic potentiation affected too few synapses to evoke homeostatic shut-down, and I wondered how many synapses of a CA1 neuron are possibly activated by extracellular stimulation in the Schaffer collateral CA3 region. Although Schaffer collateral stimulation has been a standard technique for many years, quite to my surprise, I could not find a definite answer to that question in the literature. Therefore, I have to revert to theoretical considerations: in the two-pathway experiments of the extracellular and intracellular experimental series, the two stimulation electrodes were positioned so as to activate two independent bundles of Schaffer collateral axons projecting onto the same neuron(s). Theoretically, if the population of all synapses fell into two separate, equal and non-overlapping groups, each stimulation electrode could activate maximally 50% of the total number of synapses of a neuron. Such a perfect split however seems very unlikely. Furthermore, one has to consider that of all Schaffer collateral axons, only a limited number around each stimulation electrode were activated. Estimating that not more than 10-20% of synapses on a neuron were activated by each stimulation electrode gives an upper estimate to the lower boundary. Unfortunately, the minimal number of synapses cannot be approximated this way, although this number would be more telling, as the intention of these calculations was to narrow down the range of the homeostatic threshold.

Both calculations have their limitations, of course. The calculation based on the superfusion experiments provides an upper boundary for the homeostatic threshold: if 90-98% of all synapses of a neuron are potentiated, homeostatic shut-down is turned on. A lower boundary is lacking. Again, the present experiments were not geared to provide an estimate for the homeostatic threshold. A closer approximation of this threshold can only be achieved empirically, but this is a matter for future experimental work. One would have to do a 'titration' experiment, where the proportion of potentiated synapses is systematically increased from low to high, until heterosynaptic shut-down of LTP is observed. Performing these 'titration experiments' in acute slices with the current superfusion paradigm seems unfeasible, because the recording conditions dictate the size of the superfusion spot and it cannot be varied substantially. Given that CA1 cells are contacted, on average, by only one terminal from each afferent CA3 cell (Andersen et al., 1994), a comb with many stimulation electrodes would have to be used to activate a sufficiently high proportion of afferents onto an individual cell with electrical stimulation. Recently developed microchips, where the hippocampal slice is placed onto a closely spaced array of electrodes that serve for both stimulation and recording, present another alternative (Oka et al., 1999; Besl and Fromherz, 2002).

5.6 Information processing in the hippocampus

In vitro stimulation protocols depend on experimental convenience, and the question arises, whether co-activation of a high proportion of afferents to an individual neuron within a short integration time window ever happens to a living organism.

There is evidence to support that hippocampal CA1 neurons in vivo are continuously bombarded with synaptic inputs as the hippocampus processes information involved in many forms of learning and memory (O'Keefe and Nadel, 1978; Squire, 1992; Eichenbaum and Cohen, 2001). It is therefore not unreasonable to assume that the critical number of potentiated synapses may be reached in individual neurons, even if these represent a small group of the total number of neurons activated in a specific situation. Once a high proportion of synapses are potentiated within an individual neuron that contributes to a distributed associative memory, further synaptic strengthening within that neuron has to be shut down to prevent interference and to preserve the stored information (for computational models see e.g McClelland et al., 1995; Rosenzweig et al., 2002). Under these circumstances, i.e. when new memories need to be stored without erasing old ones, it may be advantageous for fresh storage units to be supplied. This could be one of the functional roles of the proliferation of dendritic spines or even adult neurogenesis, that are both increased in the hippocampus after LTP induction or learning (Engert and Bonhoeffer, 1997; Maletic-Savatic, 1999; Nägerl et al., 2004; for review see Yuste and Bonhoeffer, 2004; Snyder et al., 2001; Deisseroth et al., 2004; for review see Kempermann, 2004).

5.7 Can synaptic plasticity shut down learning?

The idea that changes in synaptic efficacy underlie learning and memory processes is now widely accepted, although definitive proof of the 'synaptic plasticity and memory hypothesis' is still lacking (Shors and Matzel, 1997; Martin et al., 2000; Martin and Morris, 2002). If increases in synaptic strength are necessary for memory to occur, driving LTP to saturation should make further learning impossible.

My findings of homeostatic shut-down of LTP complement and extend earlier in vivo studies, which tested the proposed link between LTP and learning (for a review see Moser and Moser, 1999). The LTP-learning hypothesis predicts impairment of hippocampus-dependent learning if LTP has previously been induced in a large number of the hippocampal synapses. The saturation approach was first employed by Barnes and McNaughton. They tested whether saturation of the capacity for LTP at the perforant path/granule cell synapses of the hippocampus disrupts hippocampus-dependent spatial learning (McNaughton et al., 1986; Castro et al., 1989). LTP was induced by repeated tetanization through an electrode in the center of the angular bundle; daily sessions of tetanic stimulation resulted in cumulative LTP. When subsequently trained in circular maze or in a water maze, animals with LTP were not able to learn the location of the escape tunnel or the hidden platform, whereas animals that received low-frequency stimulation of the same pathway could. The previously tetanized animals however, were able to acquire the same task two weeks later, when LTP had decayed.

Surprisingly, attempts to replicate the disruption of learning in the water-maze failed in a number of laboratories, including the one which originally reported the effects (e.g. Robinson, 1992; Jeffery and Morris, 1993; Barnes et al., 1994). In all these attempts, the experimental procedures were similar to those followed in the original studies: high-frequency stimulation was administered on a daily basis through an electrode in the dorsal angular bundle until LTP in the perforant path synapses of the dentate gyrus reached an apparently asymptotic level. None of these replications found any evidence for an impairment of spatial learning in LTP-saturated animals relative to control-stimulated animals. However, the critical issue in any 'saturation' study will be whether a sufficient proportion of synapses has been enhanced. Thus, the failure to replicate the dramatic effects of the original studies may reflect insufficient saturation of LTP in dentate and hippocampal synapses.

The issue of incomplete saturation was addressed by a study that considerably improved the sensitivity of the saturation protocol (Moser et al., 1998). The volume of available hippocampal tissue was reduced by lesioning the hippocampus in one hemisphere. A specially designed array of concentric bipolar electrodes was implanted in the other hemisphere to increase the proportion of synapses undergoing potentiation. One bipolar stimulation electrode was placed at the medial side of the angular bundle, one at the lateral side, and one was placed in the middle. Within a single day, LTP was induced by repeated cross-bundle tetanization, using all possible combinations of tip and shaft of the two straddling electrodes. To check whether LTP was saturated in a random selection of the pathway, it was finally tested whether more LTP could be induced through the 'naïve' central stimulation electrode. Rats in which no further LTP was obtained were unable to learn the water-maze task. Non-saturated animals were comparable to a low-frequency stimulated control group.

The results of the Moser et al. study suggest that the amount of saturation is a critical factor. Learning was impaired only if the perforant path synapses had been potentiated maximally. These findings may explain why previous attempts to impair spatial learning by LTP have failed.

My experiments, assessing hippocampal CA3-CA1 LTP in an *in vitro* slice preparation, showed an analogous observation on the cellular level. The striking similarity between the *in vivo* work and my own is that a large proportion of synapses had to be saturated before any effect was noticeable, namely impairment of learning or LTP induction. Similar to the *in vivo* studies, the first step in my experiments was the saturation of LTP. In a second step, I 'probed' for LTP with a test tetanus. This in a way represents the spatial learning task the rats were tested with. I observed that further LTP was still possible unless a vast majority of synapses had been potentiated. In my experiments, I specifically tested the capacity for further LTP at heterosynaptic sites and I took great care to rule out any effects of homosynaptic occlusion. However, having been conducted in a functional context, the *in vivo* studies could not distinguish homosynaptic from heterosynaptic effects.

In their experiments, Moser and employed an elaborate stimulation pattern to achieve complete saturation of the perforant path. Their ultimate goal was to 'hit' all synapses

have no synapse left that is 'potentiable' and has not yet been potentiated. The failure to learn would thus be a consequence of homosynaptic occlusion: all plastic synapses already potentiated by electrical stimulation, no synapses would be available for the learning task to code a spatial location.

The findings presented in this study may shed new light on the behavioral results. It seems unlikely that the stimulation electrodes affected all potentiable synapses, a possibility the authors considered themselves. Accordingly, a few 'potentiable' synapses may have remained unpotentiated. Supposedly, these synapses should be able to undergo LTP and thereby permit spatial learning. My experiments however would suggest that LTP at those synapses was shut-down for homeostatic purposes. The observed inability to learn would thus be a result of heterosynaptic homeostasis.

At present, it is impossible to decide whether it was homosynaptic occlusion or heterosynaptic shut-down or a combination of both that was underlying the learning impairment in the Moser et al. experiments. In an ideal experiment, it might be possible to separate the two effects: it would require an organism with a simple behavior, a clearly defined neuronal network and a look-up table that indicates what synapse is involved in which task. Furthermore, the possibility for precise synaptic stimulation is required. One would then saturate many but not all of the synapses. Specifically, a certain population of synapses that are necessary and sufficient for a certain task should remain unpotentiated. Subsequently, one would test if the organism were able to learn that task. If learning is not possible, the impairment could unequivocally be attributed to a heterosynaptic effect. However, this is only a thought experiment and it remains to be determined whether homeostatic shut-down of LTP may influence learning on the behavioral level.

5.8 Can learning shut down synaptic plasticity?

The in vivo studies described above show that saturation of LTP can shut down subsequent learning. Is this also true the other way round, can synaptic plasticity be shut down by learning?

This question was addressed by Rioult-Pedotti and colleagues (1998; 2000; for review see Martin and Morris, 2001). They had trained rats in a skilled reaching task and then measured synaptic plasticity in vitro in brain slices of the respective primary motor cortical area. Rats learned to reach through a hole in a small plastic box with their preferred paw, and to grasp and retrieve small food pellets. After five days of daily training sessions, brain slices were prepared containing both hemispheres. Most cortical neurons lie contralateral to the limb that they control; therefore the hemisphere contralateral to the preferred forelimb was termed the 'trained' hemisphere, whereas the ipsilateral hemisphere is referred to as 'untrained', and serves as a within-subject control.

The first important finding was that, in rats that had acquired the motor skill, evoked potentials were around 50% larger in the trained than in the untrained hemisphere. The second important finding was that the learning-induced enhancement of the evoked potentials was associated with reduced LTP in the trained hemisphere. The implication is

that prior skill learning employs an LTP-like mechanism that alters the plasticity of the motor cortical synapses.

Here again, it is impossible to distinguish between homosynaptic and heterosynaptic effects. However, it is remarkable that learning of one (relatively simple) motor skill has such a pronounced effect on synaptic strength per se and on the capacity for further synaptic enhancement. This strengthens the notion that animals or humans may indeed experience situations where learning-induced plasticity drives a neuronal circuit to its limits. Therefore, neurons may have evolved strategies to cope with excess potentiation.

5.9 Homeostatic shut-down of LTP: similarities and differences to other homeostatic processes

Shut-down of LTP, as it is reported here, belongs to a large family of homeostatic processes that act in the nervous system to maintain the stability of neuronal function under ever-changing conditions. These processes are all similar in as much as their purpose is to keep neurons within their optimal operating range. There are however major differences in how they achieve their purpose: timing, target, and trigger of the respective regulatory strategy differ substantially. 'Synaptic scaling' for instance, well described in developing circuits as a reaction to altered firing rates, operates on a relatively slow timescale and affects all excitatory synapses of a neuron: their strength is multiplicatively scaled up (or down), when activity levels are decreased (or increased) (e.g., Turrigiano, 1998).

But homeostatic processes can also operate in a synapse-specific manner, e.g. through inverse heterosynaptic changes that stabilize the total synaptic weight of a neuron. Heterosynaptic depression was originally seen as a correlate of homosynaptic LTP induced in the Schaffer collateral inputs to CA1 pyramidal cells in the hippocampal slice (Lynch et al., 1977; Dunwiddie and Lynch, 1978). Tetanization of Schaffer collateral afferents lead to LTP at the synapses activated, and to LTD at different synaptic sites on the same neuron. However, heterosynaptic depression in area CA1 is observed by some groups and not by others (Scanziani, 1996; Muller et al., 1995; reviewed in Linden, 1994). In my experiments, I did not observe heterosynaptic depression, although there were one or two instances where tetanization of one pathway was accompanied by a slight transient (<five minutes) depression in the other pathway. This could be due to slightly different recording conditions in the different laboratories, as it has been shown that heterosynaptic LTD is only induced at synapses that are close enough to be sufficiently depolarized by the tetanized input (White et al., 1988; 1990).

By now, heterosynaptic depression has been reported in other preparations as well, including hippocampal dentate gyrus (Levy and Steward, 1979), hippocampal area CA3 (Bradler and Barrionuevo, 1989) and certain cortical relays (Tsumoto and Suda, 1979). Thus, the one direction of inverse heterosynaptic plasticity, namely homosynaptic potentiation accompanied by heterosynaptic depression, is well documented. However, to balance total synaptic weights also in the instance of LTD, homeostatic theory would claim that the other direction of inverse heterosynaptic plasticity operates as well. Indeed, a recent study has demonstrated heterosynaptic potentiation

for the first time (Royer and Pare, 2003). It was shown that in intercalated neurons of the amygdala, activity-dependent potentiation or depression of particular glutamatergic inputs lead to opposite changes in the strength of inputs ending at other dendritic sites. As a result, little changes in total synaptic weight occurred, event though the relative strength of inputs was modified.

Thus, inverse heterosynaptic plasticity can compensate for homosynaptic LTP and LTD. Homosynaptic and heterosynaptic plasticity is induced simultaneously by the same stimulus, and both forms of plasticity are expressed in the same time frame. Hence, the homeostatic mechanism is directly triggered by the plasticity-inducing stimulus, which causes the dual effect of synaptic strengthening (or weakening) at homosynaptic sites and synaptic weakening (or strengthening) at heterosynaptic sites, thus keeping the net change of synaptic weights roughly balanced.

The phenomenon of homeostatic shut-down that I report here is in some ways similar but in many ways also different to other homeostatic phenomena. It is similar in that – provided the required threshold is reached – it counteracts the strengthening of synapses. However, it occurs on a relatively fast timescale, it occurs in mature neuronal circuits, and it is only initiated once a certain proportion of synapses has been potentiated. Most importantly, it operates by preventing further potentiation, i.e., it leaves the basal strength of the non-potentiated synapses intact and only becomes apparent when the non-potentiated synapses are challenged by a new potentiating stimulus. Therefore, synaptic strength per se is not affected, but rather the ability of synapses to undergo further changes in synaptic strength. In that sense, homeostatic shut-down of LTP, which is a plasticity-induced effect on future plasticity events, is a form of plasticity which belongs into the general category of 'metaplasticity' mechanisms (Abraham and Bear, 1996; Warren and Tate, 1997).

The idea that synaptic plasticity is regulated by the prior history of neuronal activation relates the concept of metaplasticity to the theoretical model of Bienenstock, Cooper and Munro (BCM model, Bienenstock et al., 1982). Their model of experience-dependent synaptic plasticity was designed to account for the plasticity of visual cortex synapses during development. They proposed that synaptic modification varies as a non-linear function (Φ) of postsynaptic activity (see figure 5.4A). Low levels of activity (but above the resting level of spontaneous activity) result in LTD of the active synapses, while higher activity levels lead to LTP. The point of cross-over from LTD to LTP is termed the modification threshold Θ_M. An important aspect of the BCM model is that Θ_M is not fixed, but varies according to a time-average of prior postsynaptic activity. Therefore, Θ_M has been termed a 'sliding' modification threshold (Bear et al., 1987; Bear 1995). This is a key feature of the model, as it prevents both runaway synaptic potentiation to saturation, and conversely, a downward spiral of LTD to zero strength. The sliding threshold provides a tool of maintaining synapses within their dynamic range. For example when synapses get stronger through LTP and consequently overall afferent drive increases, the modification threshold slides to the right, making further LTP induction more difficult and LTD more likely. Conversely, when synaptic activity and postsynaptic firing are reduced, e.g. by binocular visual deprivation in developing animals, the modification threshold will slide to the left.

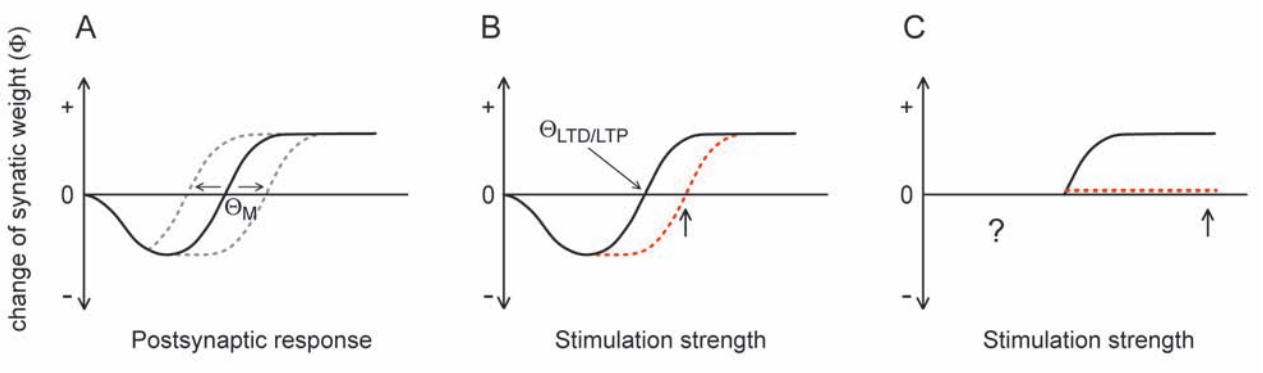


Figure 5.4 (A) Bienenstock, Cooper and Munro (BCM) model for the activity-dependent variation in the threshold for the induction of LTP. Synaptic modification may vary as a non-linear function (Φ) of postsynaptic activity (black line). The point of cross-over from LTD to LTP is termed the modification threshold Θ_M. The function Φ is not fixed, but shifts according to the history of prior activity (grey dotted lines). (B) Widespread synaptic strengthening by chemical LTP may have shifted the BCM curve and the modification threshold $\Theta_{LTD/LTP}$ in the test experiments (red dotted line). Shut-down of LTP would reflect the situation at the cross-over point of the BCM curve (arrow). (C) The test experiments may be situated at the maximal end of the BCM curve (arrow), which makes it unlikely that the shut-down of LTP can be explained by a BCM shift. Homeostatic shut-down of LTP may rather be an 'all-or-none' phenomenon, in the sense that further potentiation is completely prevented once a certain proportion of synapses has undergone LTP (red dotted line).

Metaplasticity in the most general sense denotes the phenomenon that plasticity rules themselves can be plastic. Typically, metaplasticity of LTP was studied at the same synapses that had been experimentally manipulated before (for review see Abraham and Tate, 1997). However, there is some experimental evidence that plasticity changes at one set of synapses affect subsequent plasticity at other synaptic sites (Holland and Wagner, 1998; Wang and Wagner, 1999; Abraham et al., 2001). Thus, metaplasticity is not limited to the synapses that were subject to the prior activation ('homosynaptic metaplasticity'), but can also be observed at different synaptic sites on the same cell ('heterosynaptic metaplasticity').

Metaplasticity can, for instance, result in shifting the modification threshold for LTP, making subsequent LTP induction harder (Bienenstock et al., 1982; Abraham and Tate, 1997). Can the shut-down of LTP be explained by the BCM model or is it a novel form of plasticity-induced homeostasis?

Widespread synaptic strengthening may have shifted the BCM curve and the modification threshold $\Theta_{LTD/LTP}$ in the test experiments (Fig. 4.13, Fig 5.4B). However, the tetanus used to 'probe' LTP in the test experiments had the same strength as the tetanus used in the control experiments (100Hz, 100 pulses). If a shift of the modification threshold had taken place, the same tetanus that induced normal LTP in the control experiments would then be too weak to induce LTP in the test experiments. In other words, the conditions under which shut-down of LTP was observed, would reflect the situation at the cross-over point of the BCM curve. However, whether this is an appropriate view on homeostatic shut-down is hard to judge based on the present data. In order to test whether a BCM shift underlies shut-down of LTP, one would have to explore the whole range of the curve. This could be done varying the strength of the stimulus, e.g. by systematically varying the stimulation frequency (low-frequency stimulation induces LTD while high-frequency stimulation induces LTP). If a BCM shift had taken place, induction

of LTD would be facilitated. Consequently, LTD should be observed with stimulus patterns that normally do not induce LTD (see Fig. 5.4B). A second important prediction of this model would be that the shut-down of LTP might be overcome by a stronger tetanus. While a 100Hz-tetanus failed to induce LTP, a 200Hz-tetanus should be successful. If this prediction were experimentally confirmed, it would imply that the modification threshold $\Theta_{LTD/LTP}$ is around 100Hz.

However, the postulate of a modification threshold in this frequency range renders the assumption of a BCM shift implausible, because 100Hz is far above the stimulation frequencies that have previously been reported as modification thresholds (Holland and Wagner, 1998; Wang and Wagner, 1999). Wang and Wagner showed that in rat hippocampal slices, the modification threshold $\Theta_{LTD/LTP}$ is 3Hz under normal conditions and that it can be shifted to 30Hz by prior synaptic activity. The 100Hz-tetanus that I used to test LTP is quite a strong stimulus, which normally induces clear potentiation (see Results; Wang and Wagner, 1999). Therefore, it seems more appropriate to situate my experiments at the maximal end of the BCM curve (Fig. 5.4C), which makes it unlikely, that the shut-down of LTP can be explained in terms of a BCM shift. Homeostatic shut-down of LTP may rather be an 'all-or-none' phenomenon, in the sense that further potentiation is completely prevented once a certain proportion of synapses has undergone LTP (Fig. 5.4C).

5.10 Possible mechanisms for homeostatic shut-down

What are the potential mechanisms underlying homeostatic shut-down? This is a matter for future work, but the possibilities fall into two broad categories: resource depletion or active regulation. A passive, resource depletion model would involve chemical LTP having the effect of using up limited resources necessary for LTP induction (e.g., AMPA receptors, neurotrophins). Potentiation would then automatically be shut down, even though the local conditions for triggering associative LTP induction, such as NMDA receptor activation, would occur normally. Such a model could be tested experimentally be over-expressing the molecule of interest. Increasing the supply of the molecule should prevent the shut-down of LTP. The attractive feature of a depletion model is that it achieves a homeostatic end-point without recourse to an explicit homeostatic mechanism.

Direct homeostatic regulation could operate on a network level (e.g. by increasing inhibition) or at the level of an individual neuron. For example, a reduction in excitability affecting the whole neuron might make subsequent potentiation harder (Daoudal and Debanne, 2003).

A change in the intrinsic excitability (e.g. by the regulation of ionic conductances) is a non-synaptic form of plasticity, which can influence the propagation of neuronal activity (Daoudal and Debanne, 2003). Excitability describes the predisposition of the neuron to generate an output signal – the action potential (AP) – upon a given input signal (usually an EPSP). The coupling of EPSP to AP relates the input of a neuron to its output.

It has been demonstrated that associative learning at the behavioral level leads to an increased excitability of the neurons involved and there is good evidence that there is functional synergy between synaptic and intrinsic plasticity (Daoudal and Debanne, 2003). In their landmark study, Bliss and Lømo have demonstrated that high frequency stimulation of afferents in the dentate gyrus potentiated excitatory synaptic transmission and in parallel, increased the probability that an EPSP will elicit an action potential (Bliss and Lomo, 1973). This second component has been called EPSP-to-spike potentiation (E-S potentiation). E-S potentiation was also found at the Schaffer collateral-CA1 synapse when the afferent fibers were tetanized (Andersen et al., 1980; Abraham et al., 1987; Chavez-Noriega et al., 1990). E-S potentiation is input-specific and has been demonstrated to be due (at least partly) to changes in the intrinsic excitability (Daoudal et al., 2002). Furthermore, EPSP-spike plasticity is bi-directional, similar to synaptic plasticity: the same stimulation protocol that induced LTD in area CA1 also led to a depression of the E-S coupling (Daoudal et al., 2002).

The intrinsic excitability of a neuron is defined by the properties and the distribution of ion channels in the dendritic, somatic and axonal compartments. The hyperpolarization-activated cation current (I_h) play an important role in determining membrane potential and firing characteristics of neurons and therefore is a potential target for regulation of intrinsic excitability. I_h channels are a subset of voltage-gated channels, which are permeable to both Na^+ and K^+ ions. They operate in the subthreshold voltage range where they influence membrane potential, firing threshold and firing patterns as well as synaptic integration (Hille, 2001; Magee, 1998, 1999). Furthermore, there is evidence that the regulation of I_h currents underlies the E-S potentiation or depression accompanying LTP or LTD (D. Debanne, personal communication).

Interestingly, regulation of I_h channels is also implicated in homeostatic plasticity (van Welie et al., 2004). Van Welie and colleagues performed patch-clamp recordings of CA1 neurons in hippocampal slices and demonstrated that an AMPA receptor dependent increase in synaptic activity led to an increase in somatic I_h current. Synaptic activity was enhanced non-selectively by a pharmacological boost of spontaneous neurotransmitter release in the entire slice or by directly puffing glutamate onto the recorded neuron. The authors did not describe the exact timing of their experiments, but they stated that the modulation of I_h happened on a short-timescale, within tens of minutes. As a consequence of the increase in I_h, the intrinsic excitability of the CA1 neurons was reduced. Van Welie and colleagues only studied I_h channels located at the soma. Yet, the levels of I_h in the dendrites of CA1 neurons are up to seven-fold higher than in the soma (Magee et al., 1998). Up to now, it is unknown whether the dendritic I_h channels are also modulated in response to enhanced synaptic activity. If so, increase in I_h might serve as a general cellular mechanism to dampen excitability. Thereby, the output of CA1 pyramidal neurons might be homeostatically downregulated when they receive high levels of excitatory input.

Changes in I_h current might also play a role in the homeostatic shut-down of LTP. Because the inwardly conducting I_h channels deactivate during membrane depolarizations, an effectively outward current is generated during synaptic activity (Magee, 1998; Magee

et al., 1998, Schwindt et al., 1998; Stuart and Spruston, 1998). This hyperpolarizing current shapes the EPSP and reduces temporal summation (Magee, 1998; 1999: see Fig. 5.5).

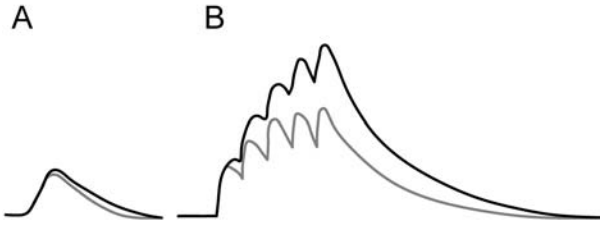


Figure 5.5 The I_h current shapes the EPSP and influences temporal summation. (A) An increase in I_h current decreases the amplitude and duration of a single EPSP and (B) reduces the temporal summation of EPSPs (grey line).

The temporal summation of EPSPs however is crucial for the induction of LTP by high-frequency tetanic stimulation, because it leads to a depolarized membrane potential which enables Ca^{2+} influx through the NMDA receptors. This in turn triggers the intracellular signaling cascades for LTP expression. Therefore, an increase in I_h might alter the potentiability of the neuron: LTP induction might be more difficult or might even be blocked completely. In my experiments, the I_h current might have been upregulated following widespread synaptic strengthening and this increase in I_h might underlie the shut-down of LTP. The involvement of I_h could be tested experimentally; for example using the paradigm of the superfusion experiments. In a first step, one would record the I_h current of CA1 neurons that had been subject to widespread potentiation and compare it to the I_h current of control neurons. This experiment would reveal whether I_h is increased following extensive LTP induction. If so, one would have to test in a second step whether it is the change in I_h that causes the shut-down of LTP. Blocking the Ih channels at the time the unpotentiated synapses are probed for LTP might be one way; alternatively, dynamic clamp technique could be employed to compensate for the increase in I_h. If an increase in I_h current was underlying the homeostatic shut-down of LTP, a block or compensation of I_h should make further potentiation possible.

An activity-dependent modulation of I_h might happen globally, altering the excitability of the entire neuron. On the other hand, I_h might also be regulated locally. It is known that increases in excitability associated with the induction of LTP are input-specific (e.g. Daoudal and Debanne, 2002), which suggests that the intrinsic excitability of different dendritic areas may be up- or downregulated differentially. Therefore, it seems conceivable that only the non-potentiated heterosynaptic sites might be affected by a homeostatic change in excitability.

Besides I_h, there are also other ionic conductances that can regulate the potentiability of neurons. A recent study proposes the involvement of a slow afterhyperpolarizing current (sI_{AHP}; Le Ray et al., 2004). To investigate the regulation of synaptic efficacy at heterosynaptic sites, Le Ray and colleagues performed sharp intracellular recordings of CA1 neurons in acute slices of rat hippocampus while two distinct Schaffer collateral pathways were stimulated (Fig. 5.6). First, a tetanus was applied to the first Schaffer collateral input to induce LTP; subsequently, the same tetanus was also applied to the second Schaffer collateral input at various time points during the experiment. When the second Schaffer collateral input was tetanized 45 minutes after the first, no LTP could be induced. However, the same tetanus applied 15 minutes later (i.e. 60 minutes after the LTP induction in the first pathway), evoked LTP (Fig. 5.6A).

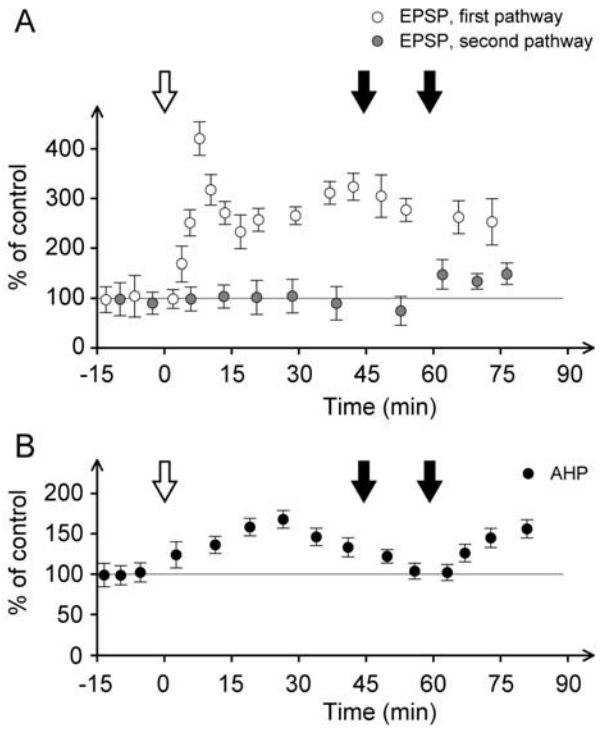


Figure 5.6 Heterosynaptic metaplasticity at Schaffer collateral-CA1 synapses. (A) Tetanization of the first Schaffer collateral pathway evoked LTP in these inputs. Tetanization applied 45 minutes later to the second pathway failed to generate LTP. When the same tetanus was reapplied to the second pathway 15 minutes later, it evoked LTP. (B) Mean peak amplitudes of the slow afterhyperpolarizing potential (sAHP) in the same experiments as in A, showing a prolonged potentiation of the sAHP. White arrow: tetanization of the first pathway, black arrow: tetanization of the second pathway. Adapted from Le Ray et al., 2004.

In some ways, the experimental approach of Le Ray et al. is similar to my extracellular and intracellular experiments. However, there are a number of aspects that are different and unfortunately, the study also has considerable weaknesses. Nevertheless, it is worthwhile to discuss their results in further detail and compare them to my observations.

It is important to note that experimental conditions were not exactly the same: The tetanization employed by Le Ray and colleagues consisted of ten 200ms/50 to 100Hz barrages repeated at 2 Hz. They authors did not state which frequency was used when and whether the same results were obtained with different frequencies. Furthermore, before a tetanus was applied, they depolarized the neuron by injecting current so that every test stimulus evoked an action potential. The input was tetanized in these conditions and then the neuron was returned to the control membrane potential. In my intracellular experiments, I did not inject depolarizing current during tetanization.

Similar to Le Ray and colleagues, I also stimulated two independent Schaffer collateral pathways to assess whether LTP induction on one pathway influences LTP on the second pathway. In my experiments, the first pathway received either a single tetanus or repeated and saturating tetani. However, I did not vary the time point of the second LTP induction, but I only 'probed' LTP after one hour. To check whether the block of LTP that Le Ray et al. have observed, can also be observed under my recording conditions, it would have been interesting to test LTP in the second pathway at earlier time-points. When I tested after one hour, I observed that LTP on the second pathway was neither blocked nor reduced. My observation is only partially consistent with the results of Le Ray and colleagues. They also observed that, LTP could be induced on the second pathway after one hour, but in their case, the amount of LTP was reduced. Le Ray and colleagues suggested that LTP on the second pathway was partially blocked by LTP on the first pathway. Unfortunately, they did not perform the relevant control experiment: testing LTP on the second pathway without previous potentiation of the first. Furthermore, I consider it a major flaw of the study that LTP on the second pathway was probed at different time points in the same experiment. In my

view, it would have been necessary to test each time-point in a separate set of slices to rule out any effect of homosynaptic metaplasticity (e.g. homosynaptic occlusion, Abraham and Bear, 1997).

In addition to recording EPSPs, Le Ray and colleagues also monitored the slow afterhyperpolarization (sAHP) at different time points during an experiment (Fig. 5.6B). They showed that Schaffer collateral tetanization triggered a potentiation of the sAHP in the CA1 neurons. Furthermore, they reported that LTP induction in the second pathway was only possible when sAHP had returned back to baseline values. The authors stated that 17 out of 32 experiments (53%) showed results as those displayed in Figure 5.6; in the other 15 cells, no clear variation in sAHP amplitude was detected although the homosynaptic LTP was present. I was puzzled that of 17 neurons, which supposedly all showed the same effect, only 5 were incorporated in the summary graphs in figure 5.6, but there was no further explanation in the paper. Le Ray and colleagues went on to show that the sAHP potentiation reduced excitability of CA1 neurons and diminished their capacity to fire prolonged spike bursts. The authors concluded that the reduced spike firing might prevent subsequent tetanization protocols to generate LTP at the nonpotentiated sites.

Based on these experiments, Le Ray and colleagues suggested that a potentiation of the sAHP might be a cellular mechanism for metaplasticity, but certainly, further experiments are required to corroborate this idea. Whether modulation of sAHP might be involved in the homeostatic shut-down of LTP that I describe, could be tested by the same kind of experiments that I proposed for assessing the role of I_h.

Altering the intrinsic excitability of a neuron is a non-synaptic way to implement homeostatic shut-down of LTP. Alternatively, the synapses might be a *direct* target of the homeostatic mechanism.

A centralistic model would propose that a 'master element' monitors, integrates and regulates the overall potentiation of a neuron. One possible scenario would be that the 'potentiation level' of the neuron is monitored in the cell body. For example, synapses might communicate their potentiation status via intra-dendritic signaling; but increased synaptic potentiation could also be detected immediately by monitoring fluctuations in membrane potential or action potential generation at the axon hillock. Upon reaching a certain potentiation level, the 'master element' would send out some 'stop-factor' (which could be a protein, but also RNA) to the dendritic tree to inhibit further potentiation. While such a central mechanism appears very stringent, it is not clear whether it may act fast enough to shut LTP down within one hour.

The potentiated synapses might also generate a 'stop-factor' themselves, via *de novo* local protein synthesis or by activation of an inactive molecule already present at the synapse. One needs to assume a concentration effect for the 'stop-factor' to account for the observation that LTP was shut down only when a large number of synapses had been potentiated. When a certain concentration at a synapse is reached, the factor might put a stop to LTP induction at that synapse. There is indeed considerable evidence for the importance of local signaling in synaptic plasticity. Intracellular calcium transients, for example, can provide a sensitive and localized readout of synaptic activity (Schulman et al., 1992; 1995). Calcium-activated enzymes, such as CamKII,

may transduce the local calcium signals to the intracellular signaling machinery. CamKII is located at the postsynaptic density and is central to the regulation of glutamatergic synapses (Lisman et al., 2002). CamKII itself, as a key player in the induction of LTP, might be the target of a homeostatic mechanism. Alternatively, CamKII may serve as detector, which activates the appropriate signaling cascades for homeostatic intervention. The potentiation capacity of a synapse can be altered without affecting basal synaptic transmission, e.g., by influencing AMPA receptor turnover or by modulating NMDA receptor properties.

Until now, it is not known whether the homeostatic mechanism for shut-down of LTP is targeted specifically to the non-potentiated synapses or whether it acts on all synapses alike. All the different regulatory mechanisms are not mutually exclusive but might reflect a different timing and rigidity of regulation that neurons may use sequentially according to the specific requirement. In contrast to resource depletion, the attractive feature of regulation is that the system might be activated in other circumstances, such as the induction of stress or other conditions in which potentiation should be stopped (Diamond et al., 2004).

5.11 Conclusion and outlook

Homeostasis is important for many biological processes; in hippocampal neurons, presumably also in neurons and networks in other brain areas, it will be an important component for controlling and shaping neuronal activity. My findings indicate that widespread synaptic strengthening on a population of neurons can prevent further potentiation of their inputs, but this shut-down is not ordinarily observed using conventional stimulation techniques. Both results are telling as they reflect two faces of the same coin: Neurons can, on the one hand, be quite robust, allow their synaptic population to regulate their efficacy relatively independently and thus sustain a substantial increase in total synaptic weight. On the other hand, neurons seem to have evolved strategies of coping with excess synaptic drive. These include, as I have shown, the homeostatic shut-down of long-term potentiation.

From introspection, we all may share the sad experience of not being able to remember the tiniest bit of the evening lecture after having sit through a day full of talks at a busy conference. And we all may have asked ourselves: “Why for heaven’s sake has my brain shut-down?” This question of course is well beyond the realm of ‘wet neuroscience’, but teaming up with other disciplines, it may once be possible to shine some light on this familiar mystery.

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Abbreviations

ACSF	artificial cerebrospinal fluid
AP	action potential
AP5	2-amino-5-phosphopentanoic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BCM	Bienenstock-Cooper-Munro
CA	cornus ammoni
CaMKII	α-Ca^{2+}-calmodulin-dependent protein kinase II
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
EPSP	excitatory postsynaptic potential
fEPSP	field excitatory postsynaptic potential
GABA	gamma amino butyric acid
I_h	hyperpolarization-activated cation current
LTP	long-term potentiation
L-LTP	long-lasting LTP
LTD	long-term depression
mEPSC	miniature excitatory postsynaptic current
MPI	Max-Planck-Institute
mRNA	messenger ribonucleic acid
NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NMDA	N-methyl-D-aspartate
NMJ	neuromuscular junction
PPF	paired pulse facilitation
sAHP	slow afterhyperpolarizing potential
STDP	spike-timing dependent plasticity
SEM	standard error of the mean
TEA	tetraethylammoniumchloride
VDCC	voltage-dependent calcium channels

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20 February 1975	Born in Frankfurt/M., Germany
August 1981 to June 1985	Elementary school, Grundschule Bieber
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October 1996	Zwischenprüfung (major subject: linguistics, minor subjects: psychology and computer science)
October 1996 to August 2000	Studies in biology at the University of Tübingen
August 1999 to June 2000	Fulbright scholarship
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