COMPARISON OF EVOKEYED SYNAPTIC RESPONSES AND SYNAPTIC PLASTICITY AT SCHAFFER COLLATERAL–CA1 AND PERFORANT PATH-CA1 SYNAPSES IN FREELY BEHAVING ADULT RATS

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Philosophiae Doctoris (PhD) in Neuroscience

of the International Graduate School of Neuroscience

Ruhr University Bochum

July 31st 2010

This research was conducted at the Department of Experimental Neurophysiology, within the Medical Faculty of the Ruhr University under the supervision of Prof. Dr. Denise Manahan-Vaughan.

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Statement

I certify herewith that the dissertation at hand was completed and written independently and without outside assistance. The "Guidelines for Good Scientific Practice" according to § 9, Sec. 3 were adhered to. This work has never been submitted in this or a similar form at this or any other domestic or foreign institution of higher learning as a dissertation.

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PhD Grade Assigned:
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<th>Full Form</th>
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<tbody>
<tr>
<td>AMPA</td>
<td>Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOAA</td>
<td>Amino-oxyacetic acid</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu Ammonis</td>
</tr>
<tr>
<td>CA1-slm</td>
<td>CA1-stratum lacunosum moleculare</td>
</tr>
<tr>
<td>CA1-sr</td>
<td>CA1-stratum radiatum</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CSD</td>
<td>Current-source density</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>E-LTP</td>
<td>Early long-term potentiation</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory post synaptic potential</td>
</tr>
<tr>
<td>fEPSP</td>
<td>Field excitatory post synaptic potential</td>
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<td>GABA</td>
<td>Gamma amino butyric acid</td>
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<td>HFS</td>
<td>High-frequency stimulation</td>
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<tr>
<td>I/O curve</td>
<td>Input/output curve</td>
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<td>LFS</td>
<td>Low-frequency stimulation</td>
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<td>LTD</td>
<td>Long-term depression</td>
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<td>LTP</td>
<td>Long-term potentiation</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>L-LTP</td>
<td>Late long-term potentiation</td>
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<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>pp</td>
<td>Perforant path</td>
</tr>
<tr>
<td>pp-CA1</td>
<td>Perforant input to the hippocampal CA1</td>
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<tr>
<td>pp-DG</td>
<td>Perforant input to the dentate gyrus</td>
</tr>
<tr>
<td>PPR</td>
<td>Paired-pulse ratio</td>
</tr>
<tr>
<td>Sc</td>
<td>Schaffer collaterals</td>
</tr>
<tr>
<td>Sc-CA1</td>
<td>Schaffer collateral input to the hippocampal CA1</td>
</tr>
<tr>
<td>STD</td>
<td>Short-term depression</td>
</tr>
<tr>
<td>STP</td>
<td>Short-term potentiation</td>
</tr>
<tr>
<td>wHFS</td>
<td>Weak high-frequency stimulation</td>
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<tr>
<td>wLFS</td>
<td>Weak low-frequency stimulation</td>
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<tr>
<td>VGCC</td>
<td>Voltage-gated calcium channels</td>
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### III. List of Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Description</th>
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<tbody>
<tr>
<td>D-AP5</td>
<td>D(-)-2-amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>Methoxyverapamil</td>
<td>(-) Methoxyverapamil hydrochloride</td>
</tr>
<tr>
<td>DCG-IV</td>
<td>(2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine</td>
</tr>
<tr>
<td>EGLU</td>
<td>(2S)-a-Ethylglutamic acid</td>
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IV. Abstract

Memory formation in the mammalian brain is believed to have its molecular basis in bidirectional persistent modifications of synaptic strength, often referred as long-term potentiation (LTP) and long-term depression (LTD). The hippocampal formation is a brain structures that is closely associated with memory formation and the expression of synaptic plasticity. In terms of its sub-regional differentiation, the hippocampal CA1 region receives cortical information directly via the perforant path (pp-CA1 synapse) and indirectly via the tri-synaptic pathway where the last relay station is the CA3 region (Sc-CA1 synapse).

The physiological properties of synaptic transmission at the Sc-CA1 synapse have been intensively studied and are well understood. On the other hand, the pp-CA1 synapse has been subjected to much less investigation, mainly done on hippocampal slices. Based on the expertise in our laboratory, the current work involved the establishment of a reliable protocol to study the pp-CA1 synapse in freely behaving animals—something, which has not yet been reported in the scientific literature. Based on this methodology, it was then possible to gain insight on the properties of basal synaptic transmission and induction of synaptic plasticity at the pp-CA1 synapse in comparison to the Sc-CA1 synapse in the intact, freely behaving adult rat.

The functional role of the pp-CA1 synapse in the hippocampal network has been approached via investigations of learning-facilitated plasticity. Here, electrically-induced short-term plasticity is facilitated into persistent long-term plasticity when induction is coupled with learning about a novel spatial environment. Previous findings point to the differential involvement of hippocampal sub-regions in learning-facilitated LTD but not LTP. For example, expression of LTP is facilitated via exposure to a novel space in the dentate gyrus (DG) and the CA1, whereas the LTD is differentially facilitated in the presence of small contextual cues in the CA1 region and large salient cues in the DG. In this study, learning-facilitated plasticity was investigated for the first time at the pp-CA1 synapse.
1. Introduction

1.1 The anatomy of the hippocampus

The hippocampus is a large structure located in the medial temporal lobe (Fig. 1.1). The name derives from the similarity in appearance of this structure with that of the marine ‘sea horse’, and was first used by the Italian anatomist, Arantius. The hippocampus proper or *Cornus Ammonis* is named after the Egyptian god Amun that has been depicted to have ram’s horns. Later on, the *Cornus Ammonis* was abbreviated as CA by Lorente de Nó and numbered from one to four to represent the sub-regions (CA1-CA4) (reviewed in Walther, 2002). Together with the dentate gyrus (DG), entorhinal cortex (EC), subiculum, presubiculum and parasubiculum the structure is mostly referred as the hippocampal formation.

![Figure 1.1. The location of the hippocampus within the rat brain.](image)

The hippocampus is a large brain structure located in the medial temporal lobe. It spans both dorsal (D) and ventral (V) parts of the rat brain. From Canto et al., 2008.

The hippocampus is tightly connected with various cortical and sub-cortical structures. The main stream of information to hippocampus is conveyed from the entorhinal cortex. The entorhinal cortex (EC) consists of 7 layers (superficial and deep layers) that comprise different cell types and anatomical properties. It is further separated, both anatomically and functionally, into lateral and medial parts, according to its afferent and efferent connections with other brain regions (Canto et al., 2008).
The superficial layers II and III of the EC connect the hippocampus through the perforant path (pp). The projections from Layer II reach the hippocampus at the level of the DG and the CA3, whereas layer III sends fibers mainly, if not exclusively, to the CA1 and the subiculum (Amaral and Witter, 1989; Witter et al., 1998, 2000; Deng et al., 2006).

The projections from EC layer II to the DG granule cells comprise the first component of the well-studied tri-synaptic pathway. The information is conveyed through the medial-pp and lateral-pp to the inner and outer molecular layer of the DG respectively. The DG projects to the CA3 via mossy fibers. The CA3 projects to the CA1 via the Schaffer collaterals. Thus, the trisynaptic pathway is named for the involvement of the three hippocampal sub-regions: DG, CA3 and CA1 (Fig. 1.3 A). The trisynaptic path to the CA1 region is diffuse and widespread in terms of connections (Amaral and Witter, 1989, 1995; Amaral et al., 1990; Ishizuka, 1990). The Schaffer collateral fibers are distributed evenly with a differentiation that involves the proximal CA3 cells, to connect the CA1 cells septal to their location, and the distal CA3 cells to more ventral levels of CA1 (Fig. 1.2) (Witter et al., 1998, 2000; Deng et al., 2006).

**Figure 1.2. The hippocampal connectivity**

The hippocampus is tightly connected with entorhinal cortex and receives direct connections from the superficial layers II and III. The layer II fibers terminate at the DG and the CA3, whereas the layer III fibers terminate at the CA1 distal dendrites. From Canto et al., 2008.

In contrast to the trisynaptic input, the direct pathway from EC layer III to the CA1 is organized in a topographical way, forming almost a one-to-one connection. Moreover, the lateral and medial pp terminates to the parts of the CA1 close to the subiculum and away from the subiculum (close to the CA3) respectively (Witter et al., 1998, 2000; Deng et al., 2006). The
direct path between the EC and the CA1 is also called the temporoammonic pathway due to its composition of temporal cortical area and Cornus Ammonis (CA) of the hippocampus.

The connections within the hippocampus are notable for their unidirectional excitatory nature. The last “stop” before the information leaves the hippocampus is the CA1 region, which is connected to the EC deep layers directly or indirectly, via the subiculum. Eventually, the information processed in the hippocampus is sent back to the EC forming a circuit between the EC and the hippocampus (Deng et al., 2006).

1.1.1 The anatomy of the CA1 sub-region

The CA1 sub-region of the hippocampus is composed of pyramidal cells (> 400 000) that are aligned in a row-like fashion at the dorsal parts. They have typically a small oblong soma and numerous basal dendrites that are short and confined to the stratum oriens below the alveus. The apical dendrites of CA1 pyramidal cells are usually few in number (1-2) that elongate towards the hippocampal fissure. The apical dendrites of the CA1 are separated in layers according to their morphology and connections (Megias et al., 2001; Spruston, 2008). The dendritic layer close to the pyramidal cell bodies is referred as the CA1-Stratum radiatum (CA1-sr) whereas the distal part of the dendrites is called stratum lacunosum moleculare (CA1-slm) (Fig. 1.3. B).

Figure 1.3. The intrinsic connections of the hippocampus and the anatomy of the CA1 region.

A. Schematic drawing of the hippocampal intrinsic connections (from Yeckel and Berger, 1998). The information is conveyed from DG to the CA3 via mossy fibers (mf) and from the CA3 to the CA1 via Schaffer collaterals (sch).

B. An example of a CA1 pyramidal cell (modified from Spruston, 2008).
CA1-sr has a branched nature with many thin dendrites that originate from the thick apical dendrite. The branching is rarefied close to the border of stratum radiatum and stratum lacunosum moleculare. On the other hand, at the CA1-slm the thick apical dendrite bifurcates to thinner dendrites that further bifurcate and are elongated above the hippocampal fissure. The spine density on the dendrites has been shown to be much less at the CA1-slm compared to CA1-sr layer (Desmond et al., 1994; Megias et al., 2001). Other morphological differences include the shape, the density and the ratio of the excitatory and inhibitory synapses at the two layers. The excitatory synapses (GABA negative terminals, asymmetrical synaptic contacts) terminate on the dendritic spines at the CA1-sr, whereas at the CA1-slm they are larger in size and contact the dendritic shafts as well as the spines. Regarding the inhibitory synapses (GABA positive terminals, symmetric synaptic contacts), the target is mostly the dendritic shaft at the CA1-sr layer, but at the CA1-slm they terminate also on spines (Megias et al., 2001). Another important feature is the higher proportion of the inhibitory synapses at the CA1-slm compared to CA1-sr (Megias et al., 2001).

These two dendritic layers of the CA1 receive afferents from different structures (Insausti et al., 1997; Kajiwara et al., 2008). The CA1-sr is known to be the terminal layer for the Schaffer collateral fibers that are mainly excitatory and synapse on many small round spines present on the dendrites, whereas the CA1-slm is the final destination for the direct path from the EC layer III and has excitatory as well as inhibitory outcomes as it activates the inhibitory network as well (Kajiwara et al., 2008).

The CA1 region additionally receives direct connections from other cortical and sub-cortical regions. It has been shown that the medial septum sends cholinergic fibers to the CA1-sr but not the CA1-slm. Conversely the perirhinal cortex (Naber et al., 1999), the basolateral nucleus of amygdala (Kemppainen et al., 2002) and nucleus reuniens thalami (Dolleman-Van der Weel et al., 1997; Vertes et al., 2006) connect to the CA1-slm and not CA1-sr.

There are several molecular differences between the CA1-sr and CA1-slm layers. For example the A-type potassium channels (Ito and Schuman, 2009) and hyperpolarization activated cation channels HCN(1) are differentially distributed in the two dendritic layers of the CA1 (Tsay et al., 2007; Komendantov and Ascoli, 2009). The molecular differentiation between the Sc-CA1 and pp-CA1 is present not only at the postsynaptic but also at the presynaptic site of the
connections. The Sc-CA1 synapse employs presynaptic P/Q-type and N-type voltage-gated Ca2+ channels activation, however the pp-CA1 synapse differ in the relatively low level of contribution from the N-type voltage gated Ca2+ channels (Ahmed and Siegelbaum, 2009). Accordingly, the probability of transmitter release has been shown to be lower for the pp-CA1 synapse compared to the Sc-CA1 synapse at basal conditions (Ahmed and Siegelbaum, 2009).

1.2 The hippocampus and learning and memory

The hippocampus is assigned a major role in learning and memory mechanisms (Squire, 1986; Nadel and Moscovitch, 1997; McGaugh, 2000; Squire et al., 2004). The famous patient HM displayed severe impairment in new memory formation due to the bilateral removal of medial temporal lobe that includes hippocampal formation (Scoville and Milner, 1957). It was shown that his impairment extended to the formation of declarative memories that could be further divided as the memory of events (episodic memory) and general knowledge about the world (semantic memory) (Scoville and Milner, 1957; Fortin and Rose, 2001; Leutgeb et al., 2005). However, he was quite successful in motoric learning as well as language skill tasks that are generally referred as non-declarative memories. Thus, the interaction with HM and the invaluable information he provided about memory function led to the first evidence that declarative and non-declarative memories are processed by distinct structures (Scoville and Milner, 1957; Eichenbaum, 2004; Eichenbaum et al., 2007).

Over the years, accumulation of the knowledge about the capabilities of HM lead to the general conclusion that hippocampus is one of the key structures in the formation of new memories. Still, the specific role of the hippocampus in memory among many other structures tightly connected in the medial temporal lobe is the subject of an ongoing debate. The main question has been related to the storage and recollection of declarative memories. Is the hippocampus important only for the formation and recollection of declarative memories or does it have capacity for storage as well (Squire, 1986; Nadel and Moscovitch, 1997; Squire et al., 2004).

In rodents, the permanent dysfunction of hippocampus impairs spatial learning acquisition as well as formation of long-term memories as assessed in navigational learning tasks such as Morris water maze or radial arm maze (Morris et al., 1989; Olton, 1983). Additionally, the hippocampus has been related with object-place associations (but not the object recognition) as
well as event-place associations (Kemp and Manahan-Vaughan, 2004; Buffalo et al., 2006). The hippocampus is also known to perform novelty detection tasks through pattern completion and pattern separation (Leutgeb and Leutgeb, 2007). Pattern separation usually refers to the ability of hippocampus to separate representations with common components. On the other hand, pattern completion is the capability to regard slightly different representations as the same (Gilbert et al., 1998; Leutgeb and Leutgeb, 2007).

1.2.1 The CA1 sub-region and learning and memory

Increasing knowledge derived from behavioural and electrophysiological research has enabled more detailed insights into the sub-regional differentiation of the role of the hippocampus in learning and memory (Gilbert et al., 2001; Kemp and Manahan-Vaughan, 2004, 2005, 2007a, 2007b, 2008; Kesner et al., 2004, 2006). The CA1 region, as the last module in the network of the hippocampus proper, has been subjected to particular attention.

The importance of the two parallel inputs to the CA1 has been studied in different species with a wide range of methods, and has been incorporated in many models of hippocampal functioning. For example, in non-human primates, the activation of the CA1-slm has been reported in response to variety of tasks with different cognitive and motoric demands (e.g. delayed match-to sample task, oculomotor delayed response task) (Sybirska et al., 2000).

The identification of the two pathways to the CA1, is a task difficult to face, as both may converge on a single CA1 cell (Kajiwara et al., 2008). A recent study by Brun and his colleagues addressed that issue in a series of experiments where they isolated the pp-CA1 synapse by chemical lesions of the CA3 sub-region or mechanical separation via a razor-blade-cut of Schaffer collateral fibers along the hippocampal septo-temporal axis. Interestingly, their results suggest that the direct entorhinal input seems to be sufficient for spatial recognition memory but not for the recall of these memories (Brun et al., 2002). Yet another approach revealed the importance of the direct entorhinal input in the long-term consolidation of the spatial memories, but not in the learning acquisition and short-term memories. Here, electrolytic lesions along the direct path from the EC to the CA1 (temporoammonic fibers) led to impairments in long-term memories as assessed 28 days after the training in Morris water maze (Remondes and Schuman, 2004).
Interestingly, preferential damage to the EC layer III neurons was observed in some patients suffering from temporal lobe epilepsy (Ang et al., 2006; Wu and Schwarcz, 1998). That finding has been used to generate an animal model of epilepsy that is induced by focal injection of amino-oxyacetic acid (AOAA) and represented by a selective cell loss in the EC layer III neurons (Denslow et al., 2001).

As the EC layer III cells are the major, if not the only, source of the direct cortical input to the CA1 region application of AOAA has been used to more specifically study the function of the pp-CA1 synapse. For example, in their study, Scharfman and his colleagues suggested that theta frequency neuronal activity in the CA1 region is significantly reduced in amplitude, although there is no detectable change in the evoked responses, due to angular bundle stimulation (Scharfman et al., 1998). The same strategy has been used by other groups to explore the capability of the pp-CA1 synapse: it was shown that CA1 place cells increase in size and have lower spatial coherence and information density after selective lesions at the EC layer III (Brun et al., 2008).

1.3 Synaptic plasticity

Synaptic plasticity is known to be the most likely mechanism that is involved in learning and memory paradigms (Bear and Malenka, 1994; Braunewell and Manahan-Vaughan, 2001; Kemp and Manahan-Vaughan, 2004; Whitlock et al., 2006). Vast amounts of work have been done to ascertain the molecular as well as behavioural correlates of synaptic plasticity and learning and memory. Although not directly proven yet, evidence on the induction and maintenance requirements of synaptic plasticity and learning suggest a tight connection between this electrophysiological event and the behavioural phenomenon (Morris et al., 1986; Morris, 1989, 2003; Manahan-Vaughan and Braunewell, 1999; Huerta et al., 2000; Li et al., 2003).

Synaptic plasticity has many different forms that are expressed in hippocampus (reviewed in Zucker and Regehr, 2002; Citri and Malenka, 2008). Some of these are ultrashort, in the range of milliseconds, (e.g. paired-pulse facilitation and depression) others are in the range of minutes (e.g. short-term potentiation and depression), hours or days (long-term potentiation and long-term depression). Other specific plasticity types also exist, such as homeostatic plasticity (Turrigano, 2007) and metaplasticity (Abraham and Tate, 1997).
Long-term synaptic plasticity presents itself in many different ways. The most extensively studied and referred forms are long-term potentiation (LTP) and long-term depression (LTD) that occur as a long-term persistent increase in synaptic efficacy and persistent long-term depression of synaptic efficacy, respectively (reviewed in Malenka and Bear, 2004; Malenka and Nicoll, 1999). Although there have been some suggestions that LTP and LTD are functions opposite to each other, recent evidences reveal distinct and rather complementary roles (Kemp and Manahan-Vaughan, 2004, 2007a). LTP as an electrophysiological phenomenon fulfills the Hebb’s criteria defined for memory formation. Namely, cooperativity, associativity and input specificity (Hebb, 1949). Cooperativity represents the ability of many weak stimulations to cooperate and induce LTP in the postsynaptic cell. The associativity is the property of the weakly stimulated synapse to express LTP in case the stimulation is paired with a strong stimulus applied to a neighboring synapse. The input specificity refers to the fact that the LTP is expressed only at the synapse that receives the stimulation (Hebb, 1949). Importantly it has been shown that not only LTP but also LTD (Manahan-Vaughan and Braunewell, 1999; Kemp and Manahan-Vaughan, 2004, 2007a) fulfills Hebb’s criteria for memory formation.

1.3.1 Long-term potentiation

LTP is typically induced in the CA1 region by a brief high frequency stimulation of the afferent fibers (reviewed in Bliss and Collingridge, 1993; Huang and Kandel, 1994; Huang et al., 1996; Malenka and Bear, 2004). However, it may also be induced by for example activation of the metabotropic glutamate receptors (reviewed in Anwyl, 1999, 2009), by paired-pulse stimulation or other manipulations of afferent fibers (Lisman, 2001). Depending on the stimulation protocol, an enhancement in synaptic efficacy is observed that has different durations ranging from hours to weeks and sometimes even longer (reviewed in Abraham, 2003; Lynch, 2004; Abraham and Williams, 2003, 2008). LTP has been shown to occur in vitro and in vivo in many brain regions (eg. hippocampus, cortex, amygdala) among which hippocampus has a special rank of being the most studied and well characterized one.

Considerable effort was spent to qualify LTP as the molecular event underlying learning and memory. Notably the same agents are capable to block LTP and cause severe impairment in spatial learning (eg. NMDA receptor blockade) (Morris et al., 1986; Morris, 1989). More recently, the role of LTP in spatial learning was strengthened by the finding that spatial
exploration induces and lowers the threshold for LTP induction in the CA1 (Manahan-Vaughan and Braunewell, 1999; Li et al., 2003; Straube et al., 2003; Uzakov et al., 2005).

1.3.2 Long-term depression

LTD is characterized as a persistent reduction in synaptic efficacy and is typically induced by low frequency stimulation of the afferent fibers. However, it also is observed upon activation of metabotropic glutamate receptors (mGluRs) (Anwyl, 2006). LTD in the hippocampus is either homosynaptic or heterosynaptic, and either dependent or independent of NMDA receptor activation (reviewed in Kerr and Abraham, 1995; Poschel and Manahan-Vaughan, 2005; Massey and Bashir, 2007). The role of LTD as a learning mechanism was neglected until reliable protocols for in vivo induction were developed in the mid-1990s (Heynen et al., 1996; Thiels et al., 1996; Manahan-Vaughan, 2000). Importantly, recent work suggests a specific role for hippocampal LTD in the encoding of novel spatial information (Manahan-Vaughan and Braunewell, 1999; Kemp and Manahan-Vaughan, 2004, 2007a; Etkin et al, 2007; Nakao et al, 2002).

1.3.3 Synaptic plasticity in the CA1 sub-region

The hippocampal CA1 sub-region is one of the best-characterized structures in terms of LTP and LTD both in vivo and in vitro. However, most of the information regarding CA1 plasticity has been gathered through studies of the Sc-CA1 synapse and very little is known about plasticity at the pp-CA1 synapse.

1.3.3.1 Synaptic plasticity in the Schaffer collateral-CA1 synapse

Synaptic plasticity at the Sc-CA1 synapse is typically studied in vitro (on hippocampal slices) and in vivo (in intact brain) by placing a stimulation electrode in the Schaffer collaterals and a recording electrode in CA1-sr dendritic layer. (Mulkey and Malenka, 1992; Bear and Malenka, 1994; Manahan-Vaughan, 2000; Manahan-Vaughan et al., 2000). As mentioned above, the input from the CA3 is abundant and widespread. Thus, it is possible to record and analyze the field excitatory post-synaptic potentials (fEPSP) that usually appear as negative going potentials with a characteristic profile and no population spike (Fig. 3.5).
LTP at the Sc-CA1 can typically be induced by high frequency stimulation (e.g. 100 Hz) (Malenka and Nicoll, 1999). The induction of LTP at the Sc-CA1 synapse depends on the activation of ionotrophic glutamate receptor NMDA that allows Ca$^{2+}$ influx leading to the activation of the Ca$^{2+}$-dependent kinases (Malenka and Bear, 2004). This phenomenon is often referred to as early long-term potentiation phase (E-LTP) (Huang et al., 1996). E-LTP can be extended to late phase LTP if protein synthesis as well as activation of the immediate early genes are involved (Frey et al., 1988, 1993; Huang et al., 1996).

On the other hand LTD is typically induced by persistent low frequency stimulation (1-5Hz) (Stanton and Sarvey, 1984; Dudek and Bear, 1992; Heynen et al., 1996; Manahan-Vaughan, 1997). In the CA1 region, the induction phase requires activation of NMDA receptors that further activates the protein phosphatases and subsequently the protein synthesis and immediate early gene activation takes place (Mulkey et al., 1993; Abraham et al., 1994; Manahan-Vaughan, 2005; Hrabetova et al., 2000).

1.3.3.2 Synaptic plasticity in the perforant path-CA1 synapse

Synaptic plasticity at the pp-CA1 synapse is difficult to study due to a variety of factors. Most experimental work is done in vitro as this is easier compared to the in vivo method. Electrophysiologically, the in vitro method benefits from the possibility of avoiding signal contamination exerted by the stimulation of perforant path (Colbert and Levy, 1992, 1993; Empson and Heinemann, 1995; Remondes and Schuman, 2002). Severing afferents from the CA3 region and dissociating the DG results in an isolated CA1 region that is free of the SC input and volume conducted currents originating from the DG (Colbert and Levy, 1992; Empson and Heinemann, 1995; Remondes and Schuman, 2002, 2004). On the other hand, in vitro research has the disadvantage that the EC input in hippocampal slices is not fully preserved and one cannot obtain functional information about the role of this structure in information processing (Colbert and Levy, 1992; Empson and Heinemann, 1995; Remondes and Schuman, 2002, 2004).

A series of experiments on CA1 slices revealed the capability and characteristics of the isolated pp-CA1 synapse in terms of synaptic plasticity (Colbert and Levy, 1992; Jones, 1993; Empson and Heinemann, 1995; Remondes and Schuman, 2002). Early reports suggested that the pp-
CA1 synapse is not capable of exhibiting synaptic plasticity under conditions of an intact inhibitory network (Colbert and Levy, 1992). However, more recent work indicated that LTP at the pp-CA1 synapse indeed occurs as a result of either theta burst stimulation or high frequency stimulation (Remondes and Schumann, 2002). Furthermore, this phenomenon is blocked by NMDA receptor antagonists and L-type Ca²⁺ channel antagonists (Remondes and Schumann, 2002). LTP at the pp-CA1 synapse is sensitive to GABA-B but not GABA-A receptors that implies the necessity of GABA release inhibition (Remondes and Schumann, 2002).

Not only LTP, but also LTD, occurs at the pp-CA1 synapse and requires NMDA receptor activation (Dvorak-Carbone and Schuman, 1999). Furthermore, LTD at the pp-CA1 synapse is not affected by the inhibition of GABA-A and GABA-B receptors (Dvorak-Carbone and Schuman, 1999b).

A primary difficulty in attempting to study the EC-CA1 synapse in vivo appears at the level of identification of the evoked responses. Although the input from the EC to the CA1 region is well organized and separated at many levels, stimulation of the perforant path in vivo inevitably activates the DG, CA3 and CA1 monosynaptically as well as the CA3 disynaptically and CA1 both di and tri-synaptically. Activation of DG and CA3 by perforant path stimulation has been shown to result in strong field excitatory postsynaptic potentials (fEPSPs), thus making the analysis of perforant path evoked CA1 fEPSPs rather complicated (Yeckel and Berger, 1990; Yeckel and Berger, 1995). For a long time, the characteristic of the EC input was thought to be mainly inhibitory however current-source density (CSD) profiles clearly indicate the presence of an excitatory input to CA1-slm that is not sufficient to induce action potentials at the cell bodies. (Leung et al., 1995; Leung et al., 1998)

Although stimulation of the angular bundle in vivo has been studied, the first interpretation of the data was attributed to the volume conduction effect from the simultaneous stimulation of the DG (Stringer and Colbert, 1994). Thus, the pp-CA1 synapse has not yet been studied in intact brain of freely moving animals.
1.4 Glutamate receptors in the CA1 region

Glutamate provides the major excitatory transmission in the brain. Glutamate has a fast transmission effect via ionotropic glutamate receptors (AMPA, NMDA and kainate receptors) and slow transmission via the metabotropic glutamate receptors (mGluRs) (reviewed in Riedel et al., 2003).

1.4.1 The ionotropic glutamate receptors: AMPA and NMDA

AMPA receptors are permeable to Na+ and K+ and when activated lead to the excitatory synaptic response via an inward current when the cell is close to its resting membrane potential. On the other hand, the activation of the NMDA receptors is more challenging as they are blocked by extracellular magnesium and open only when the cell is sufficiently depolarized. The NMDA receptors are not only sodium but also calcium permeable (reviewed in Dingledine et al, 1999; Riedel et al., 2003).

There are several molecular differences between the CA1-sr and CA1-slm layers in terms of the ionotropic glutamate receptors. For example, the ionotropic glutamate receptors AMPA and NMDA have been shown to have differential distribution along the dendritic axis of the CA1 neurons (Andrasfalvy and Magee, 2001; but Nicholson et al., 2006). The number of AMPA receptors has been shown to increase to almost two-fold at the distal dendrites (Andrasfalvy et al., 2001) however further differentiation in the synapse types might complicate the interpretation on the distribution of the AMPA receptors (Nicholson et al, 2006). In addition, evidence exists that the NMDA receptors at the CA1-slm have inwardly rectifying characteristics that imply an additional differentiation at the level of subunit composition (Otmakhova et al., 2002). The NMDA/AMPA charge ratio is higher at the perforant path compared to the Schaffer collateral path that might be attributed to efforts of minimizing the effect of the longer distance travelled by the distally activated currents (Otmakhova et al., 2002).
1.4.2 The metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) comprise three different groups of G-protein coupled receptors that are classified according to their sequence, pharmacological profile and second messenger coupling (Conn and Pin, 1997). Group I mGluRs, comprising mGluR 1 and 5, are positively coupled to phospholipase C and are predominantly postsynaptically located (reviewed in Conn and Pin, 1997). They appear to be equally important for the regulation of both LTP and LTD. Group III mGluRs (mGluR 4, 6, 7, 8) are negatively coupled to adenylyl cyclase and have been suggested to function as autoreceptors for glutamate (Conn, 1995; Johansen et al., 1995; Manzoni and Bockaert, 1995; Manzoni et al., 1995; Macek et al, 1996; Ferraguti and Shigemoto, 2006). These receptors, when activated, have a strong inhibitory effect on basal synaptic transmission and by this means alter the threshold for induction of synaptic plasticity (Manahan-Vaughan and Reymann, 1995, 1996, 1997; Manahan-Vaughan, 1997, 1998).

The group II mGluRs (mGluR2 and mGluR3) are negatively coupled to adenylate cyclase that lead to reduction of intracellular cyclic AMP (reviewed in Riedel et al., 2003). With regard to synaptic plasticity they are particularly interesting because they appear to preferentially regulate LTD (and not LTP) (Manahan-Vaughan, 1997, 1998; Kulla et al, 1999). For this reason they were subjected to particular scrutiny in this dissertation, and are therefore described in more detail here:

Group II mGluRs are located both pre- and postsynaptically on neuronal synapses (however, mGluR3 mainly on glias). Regarding the CA1 region, group II mGluRs are presynaptically localized, in dense arrangement at the CA1-slm (Shigemoto et al., 1997). Application of an mGluR2 agonist to hippocampal slices reduces synaptic transmission at the pp-CA1 synapse but not at the Sc-CA1, thereby providing a tool for pharmacological differentiation of these synapses (Speed and Dobrunz, 2009).

Group II mGluRs have been shown to have a more specific role related with LTD. Application of group II mGluR agonists enables chemically-induced LTD (Manahan-Vaughan and Reymann, 1995; Manahan-Vaughan, 1997; Huang et al., 1997; Pöschel and Manahan-Vaughan, 2005) that is blocked by antagonists of group II mGluRs (Manahan-Vaughan, 1997; Pöschel
and Manahan-Vaughan, 2005). Further, the electrically induced LTD but not LTP is impaired after single application in the CA1 region (Manahan-Vaughan, 1997) and in the DG the impairment is observed after six daily applications of group II mGluR antagonist (Altinbilek and Manahan-Vaughan, 2009).

1.5 Learning-facilitated plasticity

Long-term synaptic plasticity is induced at all subregions of the hippocampus via electrical stimulation of the respective afferent fibers (Manahan-Vaughan and Braunewell, 1999; Manahan-Vaughan, 2000; Hagena and Manahan-Vaughan, 2010). Importantly, the coupling of a spatial learning event with afferent stimulation that is sub-threshold for induction of persistent plasticity facilitates long-term plasticity (Manahan-Vaughan and Braunewell, 1999; Kemp and Manahan-Vaughan, 2004, 2005, 2007a, 2007b, 2008; Lemon and Manahan-Vaughan, 2006). Learning-facilitation of LTP is related to exploration of novel environments independent of sub-regional differences (Kemp and Manahan-Vaughan, 2004) whereas learning-facilitation of LTD requires further discrimination that is differentiated at the level of sub-regions as well as type of behavioral stimulus (Kemp and Manahan-Vaughan, 2004, 2008). For example, encoding of novel space in terms of empty holeboard (Kemp and Manahan-Vaughan, 2004) or a novel recording chamber (Li et al., 2003; Straube et al., 2003) facilitates persistent LTP in both Sc-CA1 and pp-DG synapses. On the other hand, learning-facilitated induction of LTD appears to be associated with more discrete forms of spatial stimuli (Kemp and Manahan-Vaughan, 2004, 2008). Briefly, the Sc-CA1 but not the pp-DG synapse expresses learning-facilitated LTD if the environment contains spatial arrangements of fine features (e.g. small objects within the holes of the holeboard), whereas, the presence of salient features (e.g. big visible objects in the recording environment) facilitates LTD in pp-DG but not the Sc-CA1 synapse (Kemp and Manahan-Vaughan, 2004). The phenomenon of learning-facilitated long-term plasticity appears to have different molecular requirements compared to the electrically-induced long-term plasticity (Kemp and Manahan-Vaughan, 2005, 2007a; Lemon and Manahan-Vaughan, 2006, 2009).

Whether the direct cortical path to the CA1 region engages in learning-facilitated plasticity awaits further clarification.
1.6 Aim of this Study

In this work the basic electrophysiological properties of the pp-CA1 synapse were studied and compared to the Sc-CA1 synapse in freely behaving adult rats.

Schaffer collateral-CA1 synapses have been shown to undergo both electrically-induced, as well as learning-facilitated LTP and LTD *in vivo*. Whether the pp-CA1 synapse has a similar profile is an important question that will help us understand the function of the hippocampus in synaptic information processing.

Electrophysiological recordings were conducted by stimulating the perforant path, and recording from the CA1 region in freely behaving rats. The obtained results were compared with the results from the Sc-CA1 synapse.

The basic properties of electrically-induced synaptic plasticity were studied. In addition, the question was addressed as to whether the pp-CA1 synapse expresses learning-facilitated plasticity.

To gain mechanistic insight into the mechanisms underlying synaptic transmission and synaptic plasticity in this structure, the effects of antagonism of NMDA receptors and of group II mGluRs were studied.
2 Materials and Methods

2.1 Laboratory Animals

The experiments were conducted using male Wistar rats (Charles River, Germany and Zentrale Versuchstieranstalt der Medizin (ZVM), Ruhr Universität Bochum) that were kept under standard housing conditions with a light/dark cycle of 12h (light on at 8 am) and water and food allowed ad libitum. Animals were kept in a in a temperature- (22 ± 2°C) and humidity- (55 ± 5%) controlled container (Scantainer, Scanbur BK). The animals (7-8 weeks old at the time of surgery) were implanted with electrodes and a guide cannula under anesthesia (Pentobarbital, 52 mg/kg) according to the previously established laboratory procedures (Manahan-Vaughan, 1997). The animals were group-housed before the surgery (maximum 6 per cage) and single-housed after the surgery. All the in vivo experiments were conducted after 7-10 days of the surgery to allow recovery.

2.2 Electrodes

The differentially-amplified evoked potentials were obtained via recording and stimulation electrodes referenced and grounded to superficially implanted screws on the bone. Both recording and stimulation electrodes were constructed by attaching Polyurethane-coated stainless steel wire (100 µm diameter) to a cardboard support. The monopolar recording and the bipolar stimulation electrode (1 mm inter-tip distance) were cut at approximately 6 mm in length on the side that was inserted into the brain and were fixed to rubber pin-socket connector on the other side. The impedance of the recording electrode was approximately 9 kΩ. Both recording and stimulation electrodes were referenced to stainless steel screws (1.5 mm diameter, Schließblockschrauben, Optotec, Germany) soldered to silver-coated copper wires (0.8 mm diameter).
2.3 Electrode implantation

2.3.1 Anesthesia and surgical preparation

All surgical procedures were performed under anesthesia induced by intraperitoneal (i.p.) injection of Nembutal (i.e. sodium pentobarbital, initial dose of 52 mg/kg). The Nembutal solution was prepared by dissolving 2.5 ml Narcoren® (Merial GmbH, Germany) in 97.5 ml distilled water mixed with 97.5 ml 1,2-propandiol (Merck KGaA, Germany). The depth of anesthesia was checked by tail and eyelid reflexes and additional dose of Nembutal solution was administered if necessary. Pain analgesia was implemented by means of treatment with Meloxicam (1mg/kg, sub-cutaneously) twice (once before the surgery and once 24 h after the first injection). The deeply anesthetized animals were shaved at forehead and neck. Panthenol ointment (Bepanthen, Bayer Leverkusen, Germany) was applied to the eyes to prevent dehydration. The animal was placed in a stereotaxic frame (430005-series, TSE Systems, Germany) on a tissue-covered polystyrene board and covered with thick paper tissue to avoid cooling. The head was fixed to the frame via ear and nose bars. The skull was exposed via incision, the periosteum was removed and local anesthetic Lidocaine (Xylocain® Pumpspray, Astra Zeneca GmbH, Germany) was applied. The skull was cleaned with a sharp bone-spoon and dehydrated via local application of 4% H₂O₂. Bregma was marked and the head was adjusted to have a negative slope of 1 mm between bregma and a fixed point 7 mm anterioposterior towards lambda (on the midline). The stereotaxic coordinates for the electrodes and the guiding cannula were referenced to the location of bregma in terms of anterioposterior (AP) and mediolateral (ML) distance. The calculated AP coordinates were marked onto the skull and the subsequent ML coordinates were marked unilaterally onto the right hemisphere (Fig. 2.1).

The bone was drilled (High-speed micro-drill, Fine Science Tools GmbH, Germany) with drill heads (Bohrköpfe, Hager and Meisinger GmbH, Germany) of 1.1 mm in diameter. The holes for the ground and reference electrodes were drilled on the left side of the midline approximately 2 mm anterior and 2 mm lateral to bregma for the former and 7 mm posterior 3 mm lateral to the bregma for the latter. The screws were screwed carefully not to incise the brain tissue, than fixed with cyanoacrylate glue and covered with dental acrylic (Paladur®,
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Heraeus Kulzer GmbH, Germany). The holes for the recording and stimulation electrodes and the guide cannula were drilled and the dura was gently pierced with a tip of a needle. The guide cannula (5.6 mm length, 0.8 mm diameter, Gündel Biomedical Instruments, Germany) was stereotaxically lowered via a guiding needle at coordinates 0.5 mm posterior and 1.6 mm lateral to bregma with approximate depth of 4 mm to reach the lateral ventricle. Then the guide cannula was glued to the bone and guiding needle was slowly removed.

![Figure 2.1 The sites drilled on the skull](image)

The location of the drilled holes on the rat skull. The white circles indicate the position of the ground and reference screws. The black circle is the position of the guiding cannula that reach the lateral ventricle. The grey circles are the planar position of (i) the recording electrode, (ii) the stimulating electrode for Schaffer collaterals and (iii) the stimulating electrode for the perforant path (angular bundle). Modified from (Paxinos and Watson, 1998).

### 2.3.2 Electrophysiologically-guided electrode insertion

The animals were implanted unilaterally on the right hemisphere with a monopolar recording electrode at the stratum lacunosum moleculare of the CA1 region and a bipolar stimulating electrode at the angular bundle. The recording electrode was placed above the hippocampal fissure with AP coordinates 3.0 mm posterior and 2.0 mm lateral from the bregma. The stimulation electrode was lowered at the angular bundle with coordinates corresponding to the fibers of the medial perforant path, 6.9 mm posterior and 4.1 mm lateral from the bregma (Manahan-Vaughan and Reymann, 1995). The same procedure was followed for the preparation of animals to study the Sc-CA1 synapse with the difference that the recording electrode was lowered to the CA1-sr at coordinates 2.8 mm posterior and 1.8 mm lateral whereas the stimulating electrode was placed at the Schaffer collateral fibers 3.1 mm posterior and 3.1 mm lateral with respect to the bregma. Briefly, the dura was pierced and the electrodes were
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lowered slowly in the brain tissue. The evoked fEPSP responses were monitored at different depths until a typical fEPSP was obtained.

2.4 Electrophysiological recordings

2.4.1 Experimental set-up

The *in vivo* experiments were carried out 7-10 days after the implantation of the electrodes (9-13 weeks old). Data acquisition and storage was conducted with a personal computer (Windows OS, acquisition software: Intracell, Magdeburg). The head-stage of the animal was connected to an amplifier (A-M-System 1700 differential amplifier) and stimulator (High Current Stimulus Isolator, World Precision Instruments, Germany) through a flexible ribbon cable and a swivel connector. A stimulus of a single biphasic square wave pulse of 0.2 ms duration mediated by the PC was applied to a constant current stimulator unit (A385, WPI, USA) and subsequently to the stimulation electrode with a manually selected intensity (100 µA-900 µA). The evoked response generated at the recording site was differentially amplified (100x), filtered (0.1 Hz – 10 KHz bandpass) and digitized at 10 KHz through a DA / AD converter (CED 1401plus, micro CED, Cambridge Electronic Design, UK). The waveforms were displayed and analyzed with data acquisition software (Intracell, Leibniz-Institute, Magdeburg) and stored on a hard disk drive (Fig. 2.2).
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Figure 2.2 Schematic representation of the experimental set-up.

(1) PC; (2) DA/AD converter; (3) differential amplifier; (4) constant current stimulator; (5) and (6) stimulation and recording cables connected to the socket of the freely behaving animal respectively.

2.4.2 Recordings of basal synaptic transmission and induction of synaptic plasticity

Basal synaptic transmission was recorded via stimulation of the perforant path with a stimulation intensity determined from a previously established input/output (I/O) relationship (100 µA-900 µA) for each individual animal. The stimulation intensity was increased stepwise by 100 µA for ranges of 100 µA-900 µA. The test-pulse intensity was determined as approximately the intensity that elicits 40% of the maximum fEPSP slope observed in the I/O curve. Responses were evoked by stimulating at low- frequency (0.025 Hz) with a single biphasic square wave pulse of 0.2 ms stimulus duration and 10,000 Hz sample rate. For each time-point, five records of evoked responses were averaged. The cortical encephalogram (EEG) was monitored throughout the course of each experiment. The basal synaptic transmission was recorded every 5 min (average of 5 sweeps for every time point) for 75 min and every 15 min for the subsequent 225 min. In cases when the synaptic plasticity was induced the stimulation
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Protocol was applied after 60 min of basal synaptic transmission (every 5 min) and continued for 4h. The first 3 recordings after the stimulation were taken every 5 min and switched to every 15 min afterwards.

The paired-pulse ratio has been examined for five interpulse durations (20ms, 25ms, 40ms, 50ms and 100ms) in a row separated by 40 sec. The procedure was repeated (20 min apart) five times for the low-intensity stimulation and three times for the high-intensity stimulation for every animal. Low-intensity corresponds to the stimulus intensity that elicits 40% of the maximal response obtained from the input-output relationship; high-intensity is double the low-intensity (or maximum of 400μA).

For recordings in pp-CA1: High-frequency tetanus (HFS) protocols were employed. To induce short-term potentiation (STP) single burst of 100 pulses at 100 Hz (0.1 msec stimulus duration) was applied. To induce long-term potentiation (LTP) (>4 h), four bursts of 100 pulses at 100 Hz (4 bursts of 100 stimuli, 0.1 msec stimulus duration, 10 sec interburst interval) with an intertrain interval of 5 min were given that has been reliably used to induce STP and LTP respectively at the Sc-CA1 synapse (Kemp and Manahan-Vaughan, 2004). Additionally, a weak or strong tetanus of 200Hz (3 bursts of 15 stimuli, 0.2 ms stimulus duration, 10 sec interburst interval or 10 bursts of 15 stimuli, 0.2 ms stimulus duration, 10 sec interburst interval) were applied to induce LTP and STP respectively; that are protocols extensively studied for the stimulation of the perforant path in our laboratory to date (Kulla and Manahan-Vaughan, 2000, 2008; Manahan-Vaughan and Kulla, 2003; Kemp and Manahan-Vaughan, 2004).

In all hippocampal structures studied in our laboratory to date, low-frequency stimulation (LFS) consisting of 900 or 600 pulses at 1 Hz, has been shown to be effective in eliciting LTD and STD respectively (Manahan-Vaughan, 1997; Kemp and Manahan-Vaughan, 2004; Pöschel and Manahan-Vaughan, 2007; Hagena and Manahan-Vaughan, 2010). Therefore this protocol was used to study synaptic depression at pp-CA1 synapses. During LFS the stimulus strength was raised to 70% of maximum or left at 40% of maximum stimulus strength.

The same protocols were employed previously for recordings in the Sc-CA1 synapse that enabled comparison between the two inputs.
2.4.3 Analysis of evoked potentials

The stimulation of the perforant path evoked a negative-going early field excitatory postsynaptic potential (fEPSP) with a superimposed positive component and a late negative-going fEPSP (Fig. 3.5 A) in the CA1 stratum lacunosum moleculare. The appearance and the characteristic of the positive part and the second fEPSP depended on the stimulation intensity (Fig. 3.6 A). Briefly, increasing the stimulation intensity would activate the trisynaptic path to the CA1 (DG-CA3-CA1) that complicated the analysis of the evoked response. The slope of the evoked potentials was measured as the maximum slope through the five steepest points from the beginning of the fEPSP and the first minimum (Manahan-Vaughan, 1997). The stimulation of the Schaffer collaterals resulted in a single negative going potential at the CA1 stratum radiatum (Fig. 3.2.1. B) that was analyzed for the maximum slope through the five steepest points from the beginning of the fEPSP till the first minimum point (Manahan-Vaughan, 1997).

2.4.4 Severance of the Schaffer collateral input

The Schaffer collateral input has been severed in a group of animals to isolate the pp-CA1 synapse. After the final position of recording and stimulating electrodes was found, a stainless steel cannula or thin plate (4.5mm in length) has been slowly lowered at coordinates 3.3 mm posterior and 3.3 mm lateral to bregma till the whole length is inside the brain. The tip has been fixed with cyanoacrylate glue and the whole assembly was covered with dental cement. The extent of severance was examined histologically after the completion of in vivo experiments and found to cover approximately 1 mm of the septo-temporal axis for most of the cases (Fig. 2.3)
Figure 2.3 Histological examination of the severance of the Schaffer collateral input.

Left: photographs of the Nissl-stained hippocampal slices from an animal with severed Schaffer collateral input. Right: the corresponding drawings from the atlas for easier interpretation (Paxinos and Watson, 1998). Distance from bregma as indicated. alv: alveus of the hippocampus; df: dorsal fornix; hf: hippocampal fissure; LV: lateral ventricle.

### 2.5 Drug treatment

#### 2.5.1 Administration

An outer guide cannula was implanted into the ipsilateral cerebral ventricle at the time of surgical electrode placement (see section 2.3.2). To conduct intracerebroventricular (icv) injections in freely moving rats, an injector connected to a Hamilton syringe (Hamilton Company, Nevada, USA) was inserted in the intracerebroventricular guide cannula after 5 recordings (25 min after the first recording) of basal synaptic transmission. Compound or vehicle was administered (5µl volume over 5 min; 1µl/min) after the 6th baseline recording (30 min after the first recording). Multiple drug injections were separated by 30 min. The
stimulation protocol was conducted 30 min after the last injection to allow diffusion from the lateral ventricle to hippocampus (Manahan-Vaughan et al, 1998).

### 2.5.2 Compounds

The NMDA-receptor antagonist D-AP5, D(-)-2-amino-5-phosphopentanoic acid (Tocris-Cookson Ltd., Bristol, UK; obtained from Biozol, Eching, Germany) was first dissolved in 5µl of 1N sodium hydroxide solution (NaOH), then 0.9% sodium chloride (NaCl) was added to made up a solution of 100µl volume.

The L-type voltage-gated calcium channel (VGCC) antagonist (-) methoxyverapamil hydrochloride (methoxyverapamil) (Research Biochemicals International, USA) was dissolved in distilled water and injected as 100 nmol/5µl.

The group II mGluR agonist DCG-IV (Tocris-Cookson Ltd., Bristol, UK; obtained from Biozol, Eching, Germany) was dissolved in 0.9% NaCl solution and injected as 40 ng/5µl.

The group II mGluR antagonist EGLU (Tocris-Cookson Ltd., Bristol, UK; obtained from Biozol, Eching, Germany) was used at concentration of 17.5 µg per 5 µl of 0.9% sodium chloride (NaCl). The pH has been adjusted to 7 by adding 0.2M NaOH before the final concentration was reached.

### 2.6 Behavioural experiments

#### 2.6.1 Novel holeboard and object-place constellations

Synaptic plasticity was examined in the presence of a holeboard to allow monitoring the effect of spatial changes. The holeboard comprises a grey Perspex board of 39.8 x 39.8 cm in size and contains four holes in each corner (Fig 2.4). The holes are made from the same material as the board and are 5.5 cm in diameter and 5 cm in depth. The changes of spatial features within the environment were implemented by adding small objects into the holes (3 objects, one hole left empty). The objects were selected with different shapes sizes and textures, but were small enough to fit inside the holes. The first exposure to an empty holeboard was referred to as a
novel environment exposure, whereas the presence of the small objects represented novel spatial features within the environment.

According to the chosen design, the animals were first exposed to an empty holeboard and to a holeboard with objects in separate experiments. Thus, the first experiment could be interpreted as the response to a new space and the second one would be the response to the changes in the spatial features of the previously known space.

Figure 2.4 Schematic representation of the holeboard.

(A) Empty holeboard. (B) Holeboard with objects.

2.6.2 Simultaneous electrophysiological recordings during spatial learning

For all experiments conducted using freely moving animals the subjects were placed in the recording room a day before, to allow acclimatization. The experiments were conducted in recording chambers (40 x 40 x 40 cm in size) that are open at the top. The recording chambers are made of grey Perspex except for the front wall that is removable and made of colorless Perspex. The electrodes are connected to the recording and stimulation equipment via flexible cables and swivel connector that allows animals to move in the recording chamber. The induction of persistent long-term synaptic plasticity (LTP /LTD) was carried out minimum of 7 days before the experiments with holeboard. The holeboard was inserted into the recording chamber just before the stimulation protocol starts and was removed immediately (or 15 min in the case of shorter stimulation protocols) after the stimulation.
2.7 Histological analysis

The location of the recording and stimulation electrodes as well as the guiding cannula has been verified by postmortem histological visualization. The tissue was fixed, coronal slices were obtained and Nissl stained (Bock, 1989; Manahan-Vaughan et al., 1998b). Animals with misplaced electrodes were not included in the data analysis.

2.8 Data Analysis

For the analysis of the electrophysiological data the recording from each time point was calculated as the percentage of the average of the first six recordings (pre-injection basal synaptic transmission values) for every subject individually. The graphs were constructed from the mean value of the percentages ± standard error of the mean (SEM). Between group statistics was done by two-way analysis of variance with repeated measures (ANOVA) by comparing the whole train of measurements after the stimulation. Student’s t-test was used to assess the significance level where indicated (p < 0.05 was accepted as significant).
3. Results

3.1 Establishment of electrophysiological procedures for recordings of pp-CA1 potentials in freely behaving rats

The electrophysiological evaluation of the pp-CA1 synapse has not been previously attempted by other researchers in freely behaving animals. As the methodological and research approach is therefore entirely novel, this sub-chapter will describe the strategy used to establish procedures for electrophysiological recordings of the pp-CA1 synapse, as well as the outcome of the strategy.

The depth profile of the evoked fEPSPs was recorded along a vertical tract starting from the granule cell layer of DG and decreasing the depth, stepwise, with 5min intervals up to the CA1 cell layer. The recordings were taken from a single rat under anesthesia (Fig. 3.1).

The stimulation of the medial perforant path typically evoked a large positive going field response with a superimposed population spike at the DG granule cell layer. Whereas the responses evoked at the CA1-slm are much smaller in size and usually comprise volume-conducted currents as well as long latency negative going fEPSP.
Figure 3.1 Depth profile of evoked potentials as a result of perforant path stimulation

Example of typically evoked responses *in vivo* as a result of perforant path stimulation. The recordings started at the granule cell layer of DG. The depth was decreased stepwise with 5 min intervals to let the tissue recovery. The distance between each representative recording was 160 µm. The stimulation intensity for all recordings was 200 µA. Vertical scale bar: 5mV, horizontal scale bar: 3msec.

The stimulation of the Schaffer collaterals *in vitro* has been shown to evoke a negative-going fEPSP at the CA1-sr and positive one at the CA1-slm. Conversely the stimulation of the perforant path evokes a positive-going fEPSP at the CA1-sr and negative one at the CA1-slm. To visualize if the same profile could be evoked in the intact brain an additional stimulation electrode was placed at the Schaffer collaterals and independent recordings were obtained for each stimulation site from a single recording electrode placed at CA1-slm for different distances from bregma (Fig. 3.2).
Results

Figure 3.2 Examples of the evoked potentials at the CA1-slm as response to Schaffer collateral and perforant path stimulation.

Examples of typically evoked responses in vivo at the CA1-slm as a result of Schaffer collateral (the analog traces at the left column) and perforant path stimulation (the analog traces at the right column) at three different anterio-posterior coordinates. (A), (B) and (C) left column to the analog traces represent coronal sections of hippocampal formation -3.14mm, -3.30mm and -3.80mm from bregma (Paxinos and Watson, 1998). The histological examination of the recording site was depicted as a dot on the corresponding slide (Paxinos and Watson, 1998). Only for the A (distance from bregma -3.14mm) recordings from two different animals were included. Note that the recording site for the Schaffer collateral response was not optimal and the evoked potentials had rather small amplitude and positive extending recovery phase. All of the recordings were taken from freely behaving rats. The stimulation intensity for all recordings was 200 µA. Vertical scale bar: 5mV, horizontal scale bar: 5msec for all traces. alv: alveus of the hippocampus; cg: cingulum; df: dorsal fornix; FC: fasciola cinereun; GrDG: granular layer of the dentate gyrus; hf: hippocampal fissure; Hil: hilus of the dentate gyrus; LMol: lacunosum moleculare layer of the hippocampus; Mol: molecular layer of the dentate gyrus; Or: oriens layer of the hippocampus; Py: pyramidal cell layer of the hippocampus; Rad: stratum radiatum of the hippocampus.
To characterize and isolate the evoked potentials at the pp-CA1 synapse additional strategies were tried in vivo. Theoretically, the volume conduction from the strongly activated DG granule cells can theoretically be eliminated by local deactivation close to the recording site. For this purpose, a guiding cannula, the tip of which was placed at the granule cell layer of the DG, was inserted with an angle (Fig. 3.3 A). However, it was not possible to restrict the damage caused by the cannula to such a small window that would enable obtaining meaningful evoked responses. That method could be still utilized with a smaller guiding cannula but would be extremely difficult to operate in freely behaving animals.

Another strategy that we implemented to isolate the pp-CA1 synapse was to insert a guiding cannula that reached the CA3 region and through which drug delivery was possible. Fig. 3.3 B (grey solid bar)). That method has been implemented for investigations of the subregional differentiation in the behavioral paradigms (Kesner et al., 2004). However, it did not prove to be the most suitable method for electrophysiological examinations. Deactivation of the CA3 via the reversible Na+ channel blocker (lidocaine) had a general effect on the evoked potentials further complicating the outcome, as it was not possible to strictly limit the effect of the drug away from the recording site. A possible alternative would be the use of a CA3 specific agent (e.g. kainic acid) to produce a selective lesion, but that would necessitate further effort for optimization that has not been covered in this project. In the framework of this thesis however, it was attempted to mechanically constrain the recording site from the effect of the CA3 input via chronic implantation of a stainless steel plate that cut through the Schaffer collateral fibers (Fig. 3.3 B (white dashed bar)). A similar strategy was previously implemented by cutting the Schaffer collateral fibers along the septo-temporal axis of the hippocampus with mini razor blade thus leaving the CA1 region inaccessible for the trisynaptic circuit (Brun et al., 2002). In this work we did not observe drastic changes in the evoked responses for animals with either an intact or a “CA3-constrained” hippocampus (Fig. 3.4). The main reason could be the capability of the remaining connections to engage in synaptic transmission, that is not possible to control mechanically.
Figure 3.3 Schematic representation of the attempts to isolate the pp-CA1 synapse *in vivo*.

(A) Schematic representation of a method for local deactivation of the DG cells. The sagittal cut of the hippocampus was used for better visualization. The vertical difference between bregma and lambda is denoted as 1mm that has been used for surgical preparations. The electrode is represented as a dark grey bar and the recording site as a small circle at the tip. The light grey, thick, bar indicates the cannula that should reach close to the DG granule cell layer. Although theoretically this method should be very useful, please note that the recording site is already very confined and the inclusion of the cannula is likely to introduce further damage. (B) Coronal section of the hippocampus (Bregma -3.30 mm, modified from Paxinos and Watson, 1998). Addition of an intrahippocampal cannula (light grey, thick, bar) to deactivate the CA3 neurons is not likely to damage the recording site, however limiting the drug diffusion is difficult, the overall effect of small leakages would not probably be represented as behavioral outcomes, however it would complicate the interpretation of the electrophysiological recordings. The attempt to mechanically dissociate the Schaffer collaterals (white bar with dotted contour) from the CA1 region is comparably safer than drug injection however the difficulty of specifically cutting all the afferents to the CA1-sr still remains. Please note that cannula implantation for the CA3 region also cuts the Schaffer collateral fibers, thus the two groups were pooled for analysis where applicable. alv: alveus of the hippocampus; cg: cingulum; df: dorsal fornix; FC: fasciola cinereun; GrDG: granular layer of the dentate gyrus; hf: hippocampal fissure; Hil: hilus of the dentate gyrus; LV: lateral ventricle; Mol: molecular layer of the dentate gyrus; Or: oriens layer of the hippocampus; Py: pyramidal cell layer of the hippocampus; Rad: stratum radiatum of the hippocampus (Paxinos and Watson, 1998).
Results

Figure 3.4 Comparison of evoked potentials for the pp-CA1 synapse in an intact brain and where the Schaffer collaterals were severed.

(A) Examples of evoked responses *in vivo* at the CA1-slm via stimulation of the perforant path in intact brain and (B) Schaffer collateral fibers severed case (Sc-cut group). Please note that there was not a drastic change in the evoked responses, although in some cases the distinction between the first negative and the second negative was reduced. The analog traces are from basal synaptic transmission measurements, stimulated with an intensity that elicits 40% of the maximum evoked response. Vertical scale bar: 3 mV, horizontal scale bar: 5 msec for both.

3.2 Characteristics of pp-CA1 potentials: comparison with SC-CA1 potentials

The CA1 region receives information from the entorhinal cortex both directly, and indirectly via the trisynaptic path. The trisynaptic path starts at the DG that sends information to the CA3 via mossy fibers and the CA3 connects the CA1 via Schaffer collaterals. The Sc-CA1 synapse has been extensively studied electrophysiologically, both *in vivo* and *in vitro*. The data generated has provided important information on the characteristics and functionality of the CA1 region. In contrast, the pp-CA1 synapse has been approached mostly using *in vitro* electrophysiology, or indirectly by means of neuroanatomical or pharmacological studies.

Typically, stimulation of the Schaffer collateral fibers resulted in a single negative going potential at the CA1-sr. whereas, the stimulation of the perforant path elicited a composite potential with a complex shape (Fig. 3.5 (A-B)).

In this study, the fEPSPs from pp-CA1 and Sc-CA1 synapses were evaluated for their latency (delay between the stimulation artifact and the beginning of the potential) and time-to-peak (duration of the fEPSP from the beginning to the first minimum value). For six animals per group thirty fEPSPs were obtained in blocks of five (at 0.025Hz) recorded at ca. 5 min intervals (30 min recording in total) and analyzed. The evoked responses revealed longer
latency for the pp stimulation compared to the Sc stimulation (1.48 msec ± 0.22 msec and 0.96 msec ± 0.13 msec, respectively; p < 0.0001, n=6). However, the duration of time-to-peak was shorter for the pp-CA1 synapse compared to the Sc-CA1 synapse (2.79 msec ± 0.19 msec and 4.73 msec ± 0.73 msec, respectively; p < 0.0001, n=6) (Fig 3.5 (C)).

![Figure 3.5](image)

Figure 3.5 Comparison of evoked potentials for pp-CA1 and Sc-CA1 synapses.

(A-B) Examples of typically evoked responses in vivo at the CA1-slm via stimulation of the perforant path and at the CA1-sr via stimulation of Schaffer collateral fibers (C) The pp-CA1 fEPSPs had longer latency but shorter time-to-peak duration compared to Sc-CA1 fEPSPs. The stimulation artifact is denoted by (1). The first solid line after the stimulation artifact (2) indicates the beginning of the fEPSP and the second solid line (3) indicates the peak for both traces. The dashed line (2*) on the pp evoked potential indicates the appearance of a positive going part. The latency was calculated as (2) – (1); the time to peak was calculated as (3) – (2). Additionally the pp evoked potential comprised a second negative going part. Vertical scale bar: 4mV, horizontal scale bar: 5msec.

Increasing the stimulation intensity in a stepwise manner did not affect the shape of the Sc-CA1 potentials drastically, but resulted in a steeper slope and increased amplitude. However, the same procedure increased the complexity of the pp-CA1 potentials by adding volume-conducted currents due to the stimulation of the DG and a second negative going potential due to the subsequent activation of the CA3 region (Fig. 3.6).
Figure 3.6 The input-output relationship for pp-CA1 and Sc-CA1 synapses.

Typical evoked responses from (A) the CA1-slm via perforant path stimulation and from (B) the CA1-sr via the stimulation of the Schaffer collateral fibers. Increasing the stimulation intensity stepwise (100 µA - 900 µA, step size 100 µA) complicated the analysis of the fEPSPs from the pp-CA1 synapse. The stimulation intensity was increased by 100 µA in every row for both examples. (C) The fEPSP slope was recorded as the maximum slope from the beginning of the potential to the first minimum value, the values were normalized to the slope of the fEPSP evoked by 100 µA. There was no significant difference (n=7). Vertical scale bar: 3mV, horizontal scale bar: 5 msec; for both.

Additionally, the recovery phase after the completion of the input-output curve was observed to be longer for the pp-CA1 synapse (Fig. 3.7) compared to the well-known recovery period of 30 min employed for all experiments involving the Sc-CA1 synapse in our laboratory. The input-output relationship provides a means to normalize the stimulation intensity among animals as well as experiments. The extensive research in our laboratory has shown that the protocol employed to obtain the input-output curves does not have long lasting effects on basal synaptic transmission at the Sc-CA1 synapse as examined after thirty minutes. On the other hand it has been observed that the pp-CA1 synapse does not turn to initial excitability
levels even after 135 min of completion of the input-output curve ((n=4), Student’s t-test, p < 0.05, initial stimulation with 100 µA compared to 100 µA stimulation at three different time points (ii), (iii), (iv).

Figure 3.7 pp-CA1 synapse requires longer interval for recovery to the initial excitability level after establishment of the input-output relationship.

(A) The evoked responses at the pp-CA1 synapse did not return to the initial pre-i/o values 2h following the completion of the input-output curve. (i) The stimulation intensity was increased stepwise (100 µA - 900 µA, step size 100 µA) as in Fig. 3.2.3. Evoked responses for the 3 lowest stimulus intensities (100 µA, 200 µA and 300 µA) after (ii) 45 min, (iii) 90min and (iv) 135 min of the completion of (i) (n = 4). (B) Representative analog traces for each response at (i), (ii), (iii) and (iv). The fEPSP slope was recorded as the maximum slope from the beginning of the potential to the first minimum value, the values were normalized to the slope of the fEPSP evoked by 100 µA. The breaks at the x-axis indicate the differences in time scale. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all..
3.3 Synaptic transmission and plasticity at pp-CA1 and SC-CA1 synapses

3.3.1 Ultra short-term plasticity

To access more information about the electrophysiological characteristics of the intact pp-CA1 synapse at freely behaving conditions ultra short-term plasticity was examined by the means of paired-pulse ratios for stimuli with different interpulse durations.

Stimulation of a synapse with two pulses in a very short period (typically ranging in milliseconds) results in ultra short-term plasticity that is thought to reflect mainly, but certainly not exclusively, the presynaptic properties of the synapse (reviewed in Zucker and Regehr, 2002; Thomson 2000). Previously, it has been shown in vitro that the paired-pulse response of the Sc-CA1 versus the pp-CA1 is different for juvenile rats but not young adults (Speed and Dobrunz, 2009). Whether the same relationship is present for freely behaving rats was not previously investigated.

Low-intensity stimulation revealed a similar profile of the paired pulse ratio for both pp-CA1 and Sc-CA1 synapse (Fig 3.8 A left). At high intensity stimulation, a 50 ms interpulse interval resulted in a significant difference between the Sc-CA1 and pp-CA1 animals (p < 0.05; n = 7 for Sc-CA1, n = 4 for pp-CA1) (Fig. 3.8 A right). Increasing the intensity on the other hand, drastically changed the paired pulse ratio profile for the Sc-CA1 synapse (p < 0.01; n = 7) but not as much for the pp-CA1 synapse (no significant difference, p > 0.05) (Fig. 3.8 B, left-right, respectively). Analog traces are shown for better visualization (Fig 3.9).
Figure 3.8 The paired pulse ratio is different for pp-CA1 and Sc-CA1 synapse depending on stimulus intensity.

(A) Paired pulse ratio profiles for pp-CA1 and Sc-CA1 synapses at (left) low-intensity stimulation (corresponding to the intensity necessary to evoke 40% of the maximum) and (right) high-intensity stimulation (two-fold of the low-intensity or maximum of 400 µA). (B) Paired pulse ratio profiles (left) for pp-CA1 synapse and (right) for the Sc-CA1 synapse at two intensity levels. Increasing the stimulation intensity did not result in a significant change for the pp-CA1 synapse but led to paired-pulse depression at all interstimulus intervals for the Sc-CA1 synapse. The used paired-pulse intervals were: 20ms, 25ms, 40ms, 50ms and 100ms for both groups. All the recordings were collected from freely behaving animals at awake state and sitting steadily. * indicates p<0.05; ** indicates p<0.001.
Results

Figure 3.9 Analog traces representing paired-pulse responses for Sc-CA1 and pp-CA1 synapses.

(A) Representative analog traces for the Sc-CA1 synapse obtained with (left) low-stimulus intensity, (right) high-stimulus intensity for the indicated paired pulse intervals. (B) Representative analog traces for the pp-CA1 synapse obtained with (left) low-stimulus intensity, (right) high-stimulus intensity for the indicated paired pulse intervals. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.
3.3.2 The effects of high-frequency stimulation on the pp-CA1 synapse

Previously it has been shown \textit{in vitro} that long-term potentiation is induced at the pp-CA1 synapse by application of HFS (Remondes and Schuman, 2002). Various protocols are employed to examine strengthening of the synaptic transmission. In this sub-chapter the outcome of HFS application with two different frequencies has been examined at the pp-CA1 synapse. A typical protocol that elicits LTP at the Sc-CA1 synapse \textit{in vivo} consists of single or multiple 100Hz burst(s) (Kemp and Manahan-Vaughan, 2004) that was therefore examined, for comparative purposes, at the pp-CA1 synapse. Although burst(s) of 100Hz result in robust LTP at the Sc-CA1 synapse it is generally accepted to be a weak stimulus for the perforant path (Kulla and Manahan-Vaughan, 2000). Another high frequency protocol comprising 200Hz tetanic stimulation, which is not common for Sc-CA1 synapse, is reliably used to stimulate the perforant path and elicit LTP in the DG, as previously shown in our laboratory (Kulla and Manahan-Vaughan, 2000, 2008; Manahan-Vaughan and Kulla, 2003; Kemp and Manahan-Vaughan, 2004).

The stimulation of the perforant path to CA1 synapse with a single burst of 100 pulses at 100Hz (0.1 msec stimulus duration) resulted in potentiation (mean percent of baseline $137.28 \pm 18.28 \%$) as measured 5min following the stimulation and remained detectable (mean percent of baseline $128.84 \pm 11.57 \%$) at the 24 h measurements as well. On the other hand, four bursts of 100 pulses at 100 Hz induced larger potentiation in 5min (mean percent of baseline $171.29 \pm 22.75 \%$) that approached the level of weak potentiation in about two and half hours (mean percent of baseline for wHFS versus HFS; $155.70 \pm 11.32$ versus $140.56 \pm 5.01 \%$) and remained similar for the next 24 h (mean percent of baseline $146.09 \pm 17.35 \%$). The strength of potentiation induced at the pp-CA1 synapse via weak and strong 100Hz HFS was significantly different (ANOVA, $F_{(1,108)} = 10.04$; $p < 0.05$, n = 4; Fig 3.10).
Figure 3.10 Single or multiple bursts of HFS at 100Hz elicits LTP at the pp-CA1 synapse depending on the stimulus strength.

(A) High frequency stimulation with a weak or strong tetanus of 100 Hz (1 burst (wHFS) or 4 bursts (HFS) of 100 pulses) resulted in LTP of differing initial amplitudes in freely behaving rats (n = 4). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the wHFS and HFS stimulation respectively. Representative analog traces: upper row: for the wHFS lower row: for the HFS stimulation from the same measurement points as the bar graph. (i) before stimulation, (ii) after stimulation and (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.

Application of a weak or strong tetanus of 200Hz (3 or 10 bursts of 15 stimuli, respectively) resulted in robust early potentiation with differing amplitudes (mean percent of baseline for wHFS versus HFS at 5 min after stimulation; 169 ± 18 versus 207 ± 26 %). The difference at the degree of potentiation remained for the following 4h (mean percent of baseline for wHFS
versus HFS at 240 min after stimulation; 137 ± 15 versus 207 ± 20 %) and at the 24h measurements as well (mean percent of baseline wHFS versus HFS; 117 ± 14 versus 154 ± 10 %). The strength of potentiation induced at the pp-CA1 synapse via weak and strong 200Hz HFS was significantly different (ANOVA, F_{(1, 184)} = 106.24; p < 0.001; n = 5; Fig. 3.11).

Figure 3.11 Weak or strong HFS at 200 Hz elicits LTP of differing amplitudes at the pp-CA1 synapse.

(A) High frequency stimulation with a weak or strong tetanus of 200Hz (3 bursts (wHFS) or 10 bursts (HFS)) resulted in LTP of differing amplitude in freely behaving rats (n = 4). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the wHFS and HFS stimulation respectively. Representative analog traces: upper row: for the wHFS lower row: for the HFS stimulation from the same measurement points as the bar graph. (i) before stimulation, (ii) after stimulation and (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.
3.3.3 The effects of low-frequency stimulation on the pp-CA1 synapse

Long-term depression induced by LFS at the Sc-CA1 synapse has been previously examined in vivo for different rat strains, frequencies and durations (Manahan-Vaughan, 2000). For example, application of stimuli at 1Hz for 10 minutes (600 pulses) induces robust LTD with relatively short duration (approximately 2 h) at the Sc-CA1 synapse of freely behaving Wistar rats. More precisely, the fEPSP slope of the Sc-CA1 synapse is depressed to 72 ± 9 (mean percent of baseline) 5 minutes after the stimulation and recovers only to 85 ± 5 (mean percent of baseline) after 2h (Manahan-Vaughan, 2000).

On the other hand, the previous in vitro findings by Dvorak-Carbone and Schuman (1999) suggested that pp-CA1 synapse responds more easily to LFS compared to Sc-CA1 synapses under the same conditions and stimulation protocol (1 Hz, 10 min, with the same stimulus intensity used for basal synaptic transmission measurements). As yet, LTD expression at the pp-CA1 synapse in vivo has not been studied. For reasons of comparison the stimulation protocol of 1Hz, 10 min was thus chosen to examine the effect of LFS on the pp-CA1 synapse.

Stimulation of the pp-CA1 synapse with 1Hz for 10min did not reveal any difference at the pp-CA1 evoked responses compared to the test-pulse stimulation (0.025 Hz; ANOVA, \( F_{(1, 225)} = 0.0023; p > 0.05; n = 6; \) Fig. 3.12). The average of the slope of the potentials was calculated as 95 ± 8 % (mean percent of baseline) at 5 min after the stimulation and did not change significantly.

The typical low-frequency protocol that elicits depression at both the Sc-CA1 synapse in vivo and the pp-CA1 synapse in vitro failed to induce synaptic plasticity at the present study. Thus, the protocol was modified by increasing the stimulation intensity (to evoke 70% of the maximum evoked response) during the application of the LFS. This has been shown in other studies to induce either STD or LTD (Kemp and Manahan-Vaughan, 2004). Unexpectedly, increasing the stimulation intensity potentiated the evoked responses at the pp-CA1 synapse compared to the test-pulse control (mean percent of baseline for test-pulse stimulation versus LFS at 5min; 95 ± 7 % versus 124 ± 8 %) that remained about an hour (mean percent of baseline for test-pulse stimulation versus LFS at 60 min; 109 ± 5 % versus 95 ± 2 %). The
response to the stimulation was significantly different (ANOVA, $F_{(1, 366)} = 98.28; p < 0.001; n = 9$; Fig. 3.13).

Figure 3.12 Low frequency stimulation (1 Hz, 10 min) with the same stimulation intensity used for the basal synaptic transmission fails to induce synaptic plasticity at the pp-CA1 synapse.

(A) Low frequency stimulation (1Hz, 10min) with low-intensity stimulation (to evoke 40% of the maximum evoked response) did not have any effect at the pp-CA1 synapse in freely behaving rats ($n = 6$). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the test-pulse and 1Hz stimulation respectively. Upper row: representative analog traces for the test-pulse stimulation and for the 1Hz,10 min stimulation from the same measurement points as the bar graph. (i) before stimulation, (ii) after stimulation and (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.
Figure 3.13 Low frequency stimulation (1 Hz, 10 min) with high stimulation intensity that elicits an fEPSP which is 70% of the maximum evoked response, induces potentiation at the pp-CA1 synapse.

(A) Low frequency stimulation (1Hz, 10 min) with high-intensity stimulation (to evoke 70% of the maximum evoked response) resulted in potentiation at the pp-CA1 synapse in freely behaving rats (n = 9). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the test-pulse and 1Hz stimulation respectively. Upper row: representative analog traces for the test-pulse stimulation and for the 1Hz, 10min stimulation from the same measurement points as the bar graph. (i) before stimulation, (ii) after stimulation and (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.
3.4 Effect of severance of the Schaffer collaterals on the synaptic strength of the pp-CA1 synapse

3.4.1 The effects of high-frequency stimulation on the pp-CA1 synapse under intact conditions and following severance of the Schaffer collaterals

The pp-CA1 synapse is comparably difficult to study due to the strong innervation arising from the CA3 input. Previously the synapse was successfully isolated in hippocampal slices by dissociating the DG and introducing a cut at the Schaffer collaterals (Remondes and Schuman, 2002; Dvorak-Carbone and Schuman, 2004). However, the isolation of the pp-CA1 synapse in the intact brain proves to be a difficult task, as described in section 3.1. After several attempts, the severance of the CA3 sub-region via chronic implantation of a stainless steel cannula/thin plate (section 2.4.4) was chosen to isolate the pp-CA1 synapse and observe the effect of the trisynaptic pathway on the plasticity of the pp-CA1 synapse under freely behaving conditions.

Stimulation with weak HFS (100 Hz) resulted in a comparable potentiation at the pp-CA1 synapse in the intact hippocampus and where the connection to the CA3 region was severed (mean percent of baseline for the intact versus Sc-cut animals at 5min; 157 ± 8 % versus 173 ± 15 %). After 4 h, a potentiation of smaller amplitude still remained for both groups (mean percent of baseline for intact versus Sc-cut animals at 240 min; 134 ± 8 % versus 151 ± 16 %). A similar pattern was observed after 24 h (mean percent of baseline for intact versus Sc-cut animals at 25h; 129 ± 11 % versus 122 ± 12 %). Further statistical analysis revealed no significant difference between the groups for the responses to the stimulation (ANOVA, \( F_{(1, 297)} = 0.36; p > 0.05; n = 6; \) Fig. 3.14).
Figure 3.14 High frequency stimulation (100Hz, 100 pulses) induces a similar potentiation pattern at the pp-CA1 synapse under intact conditions and where SC-CA1 collaterals were severed.

(A) High frequency stimulation with a weak tetanus of 100Hz (1 burst of 100 pulses) resulted in potentiation in freely behaving rats with intact and Sc-cut conditions (n = 6). The line breaks indicate the changes in time scale.

(B) The bar graph represents the average values of the recordings at the indicated time points for the intact and Sc-cut groups respectively. Representative analog traces from the same measurement points as the bar graph. Upper row: intact, lower row: Sc-cut, (i) before stimulation, (ii) after stimulation and (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.

Similar to high-frequency stimulation with 100 Hz (1 burst, 100pulse), stimulation at 200Hz (3 bursts, 15 pulses) did not reveal any difference in responses for the intact and Sc-cut animals. The mean percent of baseline at 5min after the stimulation increased to 159 ± 16 % versus 172 ± 10 % for the intact and Sc-cut group, respectively. After 4 h, the potentiation
still remained for both groups (mean percent of baseline for intact versus Sc-cut animals at 240 min; 152 ± 13 % versus 122 ± 9 %). Statistical analysis revealed no significant difference in the responses to the stimulation with 200Hz (ANOVA, $F_{(1, 319)} = 0.28$; $p > 0.05$; $n = 8$; Fig. 3.15).

Figure 3.15 Severance of Schaffer collateral input to CA1 does not affect wHFS (200Hz, 3 bursts) induced potentiation at the pp-CA1 synapse.

(A) High frequency stimulation with a weak tetanus of 200Hz (3 burst of 15 pulses) resulted in potentiation in freely behaving rats with intact and Sc-cut conditions ($n = 6$). The line breaks indicate the changes in time scale.

(B) The bar graph represents the average values of the recordings at the indicated time points for the intact and Sc-cut groups respectively. Representative analog traces from the same measurement points as the bar graph. Upper row: intact, lower row: Sc-cut, (i) before stimulation, (ii) after stimulation and (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.
3.4.2 The effects of low-frequency stimulation on the pp-CA1 synapse under intact conditions and following severance of the Schaffer collaterals

The low-frequency protocol that elicited change in the evoked synaptic responses at the pp-CA1 synapse in intact brain was utilized for the Sc-cut animals to study the possible effect of the Sc input on the plasticity of the pp-CA1 synapse. Interestingly, 1Hz 10 min stimulation with 70% intensity resulted in the expected depression of the evoked responses at the pp-CA1 synapse when the Sc input was severed. Compared to the test-pulse control the LFS stimulation resulted in depressed responses after 5 min of the 1Hz application (mean percent of baseline for test-pulse control versus LFS at 5min; 96 ± 6 % versus 72 ± 4 %) that is partially recovered in about two and half hours (mean percent of baseline for test-pulse control versus LFS at 150 min; 96 ± 7 % versus 83 ± 8 %). The response to the stimulation was significantly different from the test-pulse control (ANOVA, $F_{(1, 318)} = 41.36; p < 0.001; n = 8$; Fig. 3.16).
Figure 3.16 Low frequency stimulation (1 Hz, 10 min) at intensity to elicit 70% of the maximum evoked response induces depression at the pp-CA1 synapse when the Sc-CA1 input is severed.

(A) Low frequency stimulation (1Hz, 10 min) resulted in depression at the pp-CA1 synapse in freely behaving rats with severed Sc input (n = 8). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the test-pulse and 1Hz stimulation respectively. Analog traces for: upper row: intact, lower row: Sc-cut, (i) before stimulation, (ii) after stimulation and (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.
3.5 Pharmacological investigations on the basal synaptic transmission and synaptic plasticity of the pp-CA1 synapse

3.5.1 Regulation of synaptic transmission and synaptic plasticity at pp-CA1 and SC-CA1 synapses by NMDA glutamate receptors

LTP induction requires activation of the NMDA receptors at the Sc-CA1 (Morris et al., 1986; Fox et al., 2006) and pp-DG synapses (Manahan-Vaughan et al, 1998). LTP at the pp-CA1 synapse is also blocked via NMDA receptor antagonist application in vitro (Remondes and Schuman, 1999). Whether LTP at the pp-CA1 synapse has the same characteristic in vivo was examined.

Stimulation of the perforant path to CA1 synapse with wHFS at 200Hz (3 bursts of 15 pulses) resulted in an initial potentiation of the evoked response (mean percent of baseline at 5 min after the wHFS; 203 ± 16 %) that gradually decreased, but did not turn to the basal synaptic transmission level for 4 h (mean percent of baseline at 240 min after the wHFS; 141 ± 18 %). Injection (icv) of the NMDA antagonist, D-AP5 30 min prior to wHFS stimulation, restrained the potentiation of the evoked responses at all time-points for 4h (mean percent of baseline at 5 min and 240 min after the wHFS, respectively; 162 ± 15 % and 106 ± 15 %). The effect of the NMDA blocker on the wHFS-induced potentiation at the pp-CA1 synapse was statistically significant (ANOVA, $F_{(1, 183)} = 97.154; p < 0.001, n = 5$; Fig. 3.17).

L-type VGCCs contribute to LTP in the dentate gyrus (Manahan-Vaughan et al, 1998). We examined whether the inhibition of LTP at the pp-CA1 synapse could be improved if an NMDA receptor antagonist and L-type VGCC are both applied. The combined effect of the NMDA and L-type VGCC blockers on the wHFS-induced potentiation at the pp-CA1 synapse was statistically significant compared to the vehicle injection (ANOVA, $F_{(1, 143)} = 49.58; p < 0.001, n = 5$; Fig. 3.18). Furthermore, the additional block of L-type VGCC inhibited the LTP at the pp-CA1 synapse significantly more compared to application of D-AP5 alone (ANOVA, $F_{(1, 143)} = 7.12; p < 0.01, n = 5$).
Figure 3.17 Weak high-frequency stimulation (200 Hz) induced potentiation at the pp-CA1 synapse is significantly inhibited by D-AP5.

(A) High frequency stimulation with a weak tetanus of 200Hz (3 bursts of 15 stimuli, 0.2 ms stimulus duration, 10 sec interburst interval elicited LTP with a smaller amplitude in the presence of NMDA blocker (D-AP5, 5µl, icv.) (n = 5). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the vehicle and D-AP5 groups respectively. Analog traces for: upper row: vehicle, lower row: D-AP5, (i) before stimulation, (ii) after stimulation and (iii) 4h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.
Figure 3.18 L-type VGCCs contribute to the NMDA receptor independent component of whFS (200 Hz) induced potentiation at the pp-CA1 synapse.

(A) Injection of L-type VGCC antagonist (methoxyverapamil) prior to D-AP5 inhibited LTP at the pp-CA1 synapse (n = 5). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the vehicle group and D-AP5+Methoxyverapamil group. Representative analog traces from the same measurement points as in the bar graph. Upper row: vehicle, lower row: D-AP+Methoxyverapamil, (i) before stimulation, (ii) after stimulation and (iii) 4h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all
3.5.2 Regulation of synaptic transmission at pp-CA1 synapses by group II metabotropic glutamate receptors.

Activation of group II mGluRs has been previously shown to depress basal synaptic transmission at the pp-CA1 synapse at concentrations that have no effect on the Sc-CA1 synapse *in vitro* (Speed and Dobrunz, 2009). In this study, the agonist action of DCG-IV on group II mGluRs was employed to characterize the sensitivity of the pp-CA1 synapse.

The injection of DCG-IV showed a rapid depression effect on basal synaptic transmission of the pp-CA1 synapse, compared to vehicle controls (mean percent of baseline for vehicle control versus DCG-IV at 5 min after injection; 96 ± 5 % versus 75 ± 5 %). The effect persisted for approximately 90 min (mean percent of baseline for vehicle control versus DCG IV; 94 ± 4 % versus 79 ± 3 %) and gradually diminished in 180 min (mean percent of baseline for vehicle control versus DCG IV; 91 ± 7 % versus 83 ± 5 %). The effect of DCG-IV injection was significant compared to the vehicle control (ANOVA, $F(1, 230) = 39.00; p < 0.001$, $n = 5$; Fig. 3.19).

 Activation of group II mGluRs reversibly depressed basal synaptic transmission at the pp-CA1 synapse. Unexpectedly, application of group II mGluR antagonist (EGLU) at concentrations, that does not affect the basal synaptic transmission in Sc-CA1 (Manahan-Vaughan, 1997) and pp-DG (Manahan-Vaughan, 1997) synapses, also depressed the basal synaptic transmission at the pp-CA1 synapse.

The depressive effect of EGLU began about 10 min after injection, peaked in about 1 h and vanished in 2 h (mean percent of baseline for vehicle control versus EGLU before injection, 101 ± 2 % versus 109 ± 11 %; 10 min after injection, 96 ± 2 % versus 74 ± 9 %; 60 min after injection 91 ± 7 % versus 73 ± 2 %; 120 min after injection 90 ± 3 % versus 95 ± 10 %; $n = 4$, Fig. 3.20).
Figure 3.19 Activation of group II metabotropic glutamate receptors reduces the basal synaptic transmission at the pp-CA1 synapse.

(A) Injection of the group II mGluR agonist DCG-IV rapidly depressed the basal synaptic transmission at the pp-CA1 synapse compared to vehicle control conditions (n = 5). The line breaks indicate the changes in time scale.

(B) The bar graph represents the average values of the recordings at the indicated time points for the vehicle and DCG-IV injected groups. Analog traces for: upper row: vehicle, lower row: DCG-IV, (i) before injection, (ii) after injection (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec for all.
Figure 3.20 Antagonism of group II metabotropic glutamate receptors reduces basal synaptic transmission at the pp-CA1 synapse.

(A) Injection of the group II mGluR antagonist EGLU depressed basal synaptic transmission at the pp-CA1 synapse compared to vehicle control conditions (n = 4). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the vehicle and EGLU injected groups. Analog traces for: upper row: vehicle, lower row: EGLU, (i) before injection, (ii) after injection (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec for all.
3.6 Learning-facilitated plasticity in pp-CA1 synapses

Learning-facilitated plasticity reflects the ability of hippocampal synapses to exhibit a facilitation of synaptic plasticity when an animal spatially learns in a novel environment. Exploration of a novel environment in the form of an empty holeboard facilitates the effect of the high-frequency stimulation and results in prolonged LTP compared to electrical stimulation alone for the Sc-CA1 and pp-DG synapses (Kemp and Manahan-Vaughan, 2004). The same paradigm was employed for the pp-CA1 synapse in the present study.

Interestingly, exploration of novel empty space during 100Hz high-frequency stimulation protocol did not facilitate long-term potentiation at the pp-CA1 synapse unlike the Sc-CA1 and pp-DG synapses. The effect of the novel space was not statistically significant compared to electrically induced potentiation at the pp-CA1 synapse (ANOVA, $F_{(1, 230)} = 0.51; p > 0.05$, $n = 6$; Fig. 3.21). This may suggest that the stimulation protocol was inadequate, or that pp-CA1 synapses are distinct from other hippocampal synapses.

Exploration of novel space containing contextual cues in the form of small objects facilitates the expression of LTD and partially inhibits LTP at the Sc-CA1 synapse (Kemp and Manahan-Vaughan, 2002, 2004). On the other hand, plasticity at the pp-DG synapse is not affected by the contextual cues (Kemp and Manahan-Vaughan, 2002, 2004).

In the present work, exposure to contextual cues facilitated both the early and late phases of LTP (mean percent of baseline for no holeboard control versus holeboard with objects: before stimulation, $97 \pm 3 \%$ versus $99 \pm 5 \%$; 5 min after stimulation, $157 \pm 21 \%$ versus $231 \pm 14 \%$; 240 min after stimulation $135 \pm 11 \%$ versus $200 \pm 14 \%; 24h$ after stimulation $149 \pm 14 \%$ versus $169 \pm 19 \%$). Statistical analysis revealed a highly significant difference between groups (ANOVA, $F_{(1, 230)} = 132.04; p < 0.001$, $n = 6$; Fig. 3.22).

In addition, the exposure to contextual cues in the presence of LFS was tested for the pp-CA1 synapse. Considering the observation that LFS (1Hz, 10 min) induces depression at the pp-CA1 synapse only when the Sc is severed, and does not occur if the hippocampus is intact, a group of Sc-cut animals was used.
Exposure to contextual cues had little effect on the early phase of the LFS-induced depression but had an inhibitory effect on LTD that became evident roughly 30 min after stimulation (mean percent of baseline for no holeboard control versus holeboard with objects: before stimulation, 100 ± 3 % versus 101 ± 3 %; 5 min after stimulation, 71 ± 5 % versus 84 ± 5 %; 30 min after stimulation 79 ± 6 % versus 87 ± 5 %; 240 min after stimulation 69 ± 8 % versus 95 ± 10 %; 24h after stimulation 71 ± 11 % versus 94 ± 13 %). The between-group statistical analysis was highly significant (ANOVA, F(1, 270) = 69.05; p < 0.001, n = 7; Fig. 3.23).

Figure 3.21 Exposure to empty holeboard has no facilitatory effect on the high-frequency stimulation at the pp-CA1 synapse.

(A) High frequency stimulation 100Hz in the presence of a novel environment resulted in LTP that is not significantly different from LTP induced by electrical stimulation only (n = 6). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the HFS alone (no holeboard) and HFS in the presence of an empty holeboard (empty holeboard)
Results

groups respectively. Representative analog traces from the same measurement points as the bar graph. Upper row: no holeboard, lower row: empty holeboard, (i) before stimulation, (ii) after stimulation and (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.

Figure 3.22 Exposure to an object-containing holeboard facilitates the effect of the HFS at the pp-CA1 synapse.

(A) Exploration of small objects partially concealed within the holeboard holes during the HFS (100Hz) facilitated the electrically induced potentiation at the pp-CA1 synapse (n = 6). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the HFS alone (no holeboard) and HFS in the presence of a holeboard containing objects (holeboard with objects) groups respectively. Representative analog traces from the same measurement points as the bar graph. Upper row: no holeboard, lower row: holeboard with objects, (i) before stimulation, (ii) after stimulation and (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.
Figure 3.23 Exposure to an object-containing holeboard inhibits synaptic depression when the Schaffer collateral input is dissociated.

(A) Exploration of fine spatial cues reversed the synaptic depression elicited by low-frequency stimulation (1Hz, 10min) at the pp-CA1 synapse (n = 5). The line breaks indicate the changes in time scale. (B) The bar graph represents the average values of the recordings at the indicated time points for the LFS stimulation (no holeboard) alone and exposure to holeboard with small objects (holeboard with objects) respectively. Representative analog traces from the same measurement points as the bar graph. Upper row: no holeboard, lower row: holeboard with objects, (i) before stimulation, (ii) after stimulation and (iii) 24h measurement. Vertical scale bar: 3mV, horizontal scale bar: 5msec; for all.
4 Discussion

4.1 Summary

In this study, electrophysiological procedures were established in freely behaving adult rats to study the direct cortical input from the entorhinal cortex to the hippocampal CA1 sub-region.

The electrophysiological characteristics of the evoked responses at the pp-CA1 synapse were studied in comparison to the well-known Sc-CA1 synapse. Compared to the Sc-CA1 synapse, the pp-CA1 synapse was observed to comprise a longer latency and a shorter time-to-peak duration as well as aberrant contamination from the activation of the tri-synaptic pathway. Furthermore, the two synapses at the CA1 sub-region responded differentially to paired-pulse stimulation, depending on the intensity of stimulation.

High-frequency stimulation (HFS) elicited potentiation at the pp-CA1 synapse the amplitude and duration of which depended on the frequency and strength of the protocol used. On the other hand, application of a typical low-frequency stimulation, that has been known to elicit persistent depression at the Sc-CA1 synapse, either failed to induce plastic changes at the pp-CA1 synapse or resulted in potentiation of the evoked responses depending on the intensity of stimulation.

The severance of the input from the CA3 sub-region had no impact on the results of high-frequency stimulation but reversed the outcome of the low-frequency stimulation (enabling LTD) suggesting a modulatory role of the tri-synaptic input on the plasticity of the pp-CA1 synapse.

Pharmacological approaches revealed the contribution of both the NMDA receptor and the voltage gated calcium channels for the HFS- evoked synaptic potentiation at the pp-CA1 synapse. Basal synaptic transmission was not affected by antagonism of NMDA receptors but was depressed by activation (or pharmacological antagonism) of group II metabotropic glutamate receptors.
Learning-facilitated plasticity was also examined at the pp-CA1 synapse. In contrast to previous findings for the Sc-CA1 and pp-DG synapses, the exploration of a novel environment did not facilitate the expression of long-term potentiation at the pp-CA1 synapse. On the other hand, exploration of a novel-object-containing holeboard significantly facilitated the response of the pp-CA1 synapse to high-frequency stimulation and reversed LTD.

The results of the present work suggest distinct properties of the pp-CA1 synapse in the expression of electrically-induced synaptic plasticity, and a role in learning-facilitated plasticity.
4.2 Detailed discussion

4.2.1 Challenges in obtaining pp-CA1 evoked potentials in vivo

The significance of the direct input from the entorhinal cortex to the CA1 has been acknowledged in many ways over the past few decades (Lisman, 1989; Treves and Rolls, 1994; 2004; Jarrard, 1993; McClellan et al., 1995; Rolls, 1996; Hasselmo et al., 2002; Rolls and Kesner, 2006). Anatomically its presence has been made known from the detailed studies reported by Ramon y Cajal (1911) and Lorente de No (1934) and was later confirmed by others (Steward 1976; Witter et al., 1988, 2000; Canto, 2008). The electrophysiological characterization of the synapse however has been subject of a debate for a long time (Jones et al., 1993; Soltesz, 1995; Soltesz and Jones, 1995). The early in vivo attempts to record from the pp-CA1 synapse revealed difficulties in obtaining a short-latency monosynaptic EPSP that was mainly attributed to the strong inhibitory control over the distal dendrites of the CA1 in rats (Soltesz, 1995; Soltesz and Jones, 1995). However, a clear monosynaptic excitatory activation at the CA1 has been reported in studies on rabbits (Yeckel and Berger, 1990; 1995) leaving the open question as to the excitatory-inhibitory relationship of the synapse. In general, it is now accepted that indeed it is possible to evoke a short latency monosynaptic EPSP at the CA1-slm via the stimulation of the angular bundle from an intact rat brain (Stringer and Colbert, 1994; Leung, 1995).

To further complicate the picture, the volume conduction from the strongly activated DG (and possibly the CA3) has been described as a direct concomitant of the monosynaptically-recorded EPSPs from the CA1 (Stringer and Colbert, 1994). The influence of the DG can be eliminated via current-source density (CSD) analysis that reveals the local synaptic activation independent of volume conduction (Canning and Leung, 1997; Canning et al., 2000; Leung et al., 1995; Leung, 1995). Current-source density analysis for hippocampal neurons is applied, assuming one directional (vertical) current flow through the dendritic tree. That appears to be a good approximation, since the dendrites are vertically aligned towards the hippocampal fissure for both the CA1 and the DG. Typically used, is a 2nd-order differencing formula for evoked potentials, recorded with a fixed distance on a single vertical tract that spans the dendritic regions of the CA1 and the DG (Canning and Leung, 1997; Canning et al., 2000;
Leung et al., 1995). CSD analysis appears to be a very successful method that, however, has limits: It neglects the volume conduction in the lateral plane and depends on the conduction of the tissue that could be changed from spot-to-spot, not only depending on the evoked responses, but also on the possible tissue movements and/or damage introduced by the electrode insertion. CSD analysis is a demanding procedure that depends on multiple recordings that can be achieved by increasing or decreasing the depth of a single recording electrode with a fixed step size, which could be reliably performed in anesthetized rats, but not under freely behaving conditions. Recently, the development of multichannel electrodes allows simultaneous recordings from many locations that can be successfully applied under anesthetized conditions, and make the application of the method in freely behaving animals possible. This would, however, require a different technical set-up (Bragin et al., 2000; Townsend et al., 2002; Cheung, 2007) and has not been used in our laboratory. The in vitro studies mechanically circumvent the contamination on the pp-CA1 synapse introduced by the activation of DG. It has been well documented that cutting the CA3 region and dissociating the DG leaves an isolated CA1 region that enables the electrophysiological characterization of the pp-CA1 synapse (Remondes and Schuman, 2003; Speed and Dobrunz, 2009).

The challenges in obtaining pp-CA1 evoked potentials in vivo start at the anatomical level. The direct connections from the EC to the CA1 appear to be relatively confined following a topographical, almost one-to-one pattern (Tamamaki and Nojyo, 1995; Witter et al., 1988, 2000; Deng et al., 2006). The divergence starts at the origin of the pathway as the fibers from the lateral entorhinal cortex terminate mainly on the parts of CA1 stratum lacunosum moleculare that are close to the CA3 region, whereas the lateral perforant path mostly terminates on the CA1-slm components that are close to the subiculum (Fig. 1.2). Thus, it appears of importance to have a relatively precise location for both recording and stimulating electrode. On the other hand, the Schaffer collateral input to the CA1-sr has a massive nature that enables relatively well defined procedure to find optimal recording-stimulation coordinates (Amaral and Witter, 1989, 1995; Amaral et al., 1990, 1993; Ishizuka et al., 1990, 1995).

The in vitro studies on the pp-CA1 synapse, typically aim at part of the CA1-slm as the stimulation zone, since the fibers mostly transverse close to the recording site (Otmakhova and Lisman, 1999, 2000; Remondes and Schuman, 2002, 2003, 2004). In vitro preparations
benefit from a relatively confined region of stimulation and the excited terminals are limited. However, they suffer the likelihood of stimulating the other direct inputs innervating the CA1-slm (e.g. nucleus reuniens thalami) (Wouterlood, 1990; Bertram and Zhang, 1999). Another aspect of the in vitro preparations comprises the necessity to preserve as many intact fibers as possible, which might be difficult due to the slicing procedure (Empson and Heinemann, 1995). On the other hand, the stimulation of the perforant path in vivo is possible via placing a stimulating electrode at the angular bundle (Stringer and Colbert, 1994; Leung et al., 1995; Kulla and Manahan-Vaughan, 1998). The in vivo studies conducted on an intact brain are not likely to lack a portion of the afferent fibers. The stimulation of the angular bundle (at the coordinates we and others used (Leung et al., 1995, Manahan-Vaughan, 1998) is not expected to activate the direct thalamic innervation to the CA1-slm but it is not possible to differentiate the tract originating from the EC layer III (that mainly if not exclusively innervates the CA1-slm directly (Witter et al., 1998, 2000) and the tract from the EC layer II, that is the direct source for DG and CA3 (Amaral et al., 1990; Amaral and Witter, 1989, 1995). Furthermore, the angular bundle comprise the medial perforant path as well as the lateral perforant path fibers (Amaral et al., 1990; Amaral and Witter, 1989, 1995) and it might be not possible to exclusively select the former or the latter one. In the present study, we employed stimulation coordinates that enable electrode placement in the medial perforant path that has been studied extensively by our group, and others, in the case of DG functionality (Leung et al., 1995; Manahan-Vaughan, 1998; Frey and Frey 2009).

The recording site for the pp-CA1 synapse comprises the distal dendritic layer (CA1-slm) that is typically half, or one-third, the size of CA1-sr, where the Schaffer collateral fibers terminate (Amaral and Witter, 1989; Amaral et al., 1990, 1995; Witter et al., 1988, 2000). Consequently to restrict the recording site to CA1-slm methodologically is difficult due to the size of the extracellular recording electrodes. Furthermore, the anterioposterior and mediolateral coordinates of the recording site for the pp-CA1 synapse are also subject for optimization due to the particular distribution of the afferents from the medial and lateral EC at the septo-temporal axis of the CA1 region. Thus, in the first part of this work we aimed to establish a reliable protocol for the in vivo recordings from the freely behaving rats.
4.2.2 Comparative characteristics of evoked potentials at pp-CA1 and SC-CA1 synapses

The laminar alignment of the hippocampal pyramidal cells, with dendrites elongating in one direction, provides the beautiful appearance that captured the attention not only of anatomists but also electrophysiologists for many decades (Spruston, 2008). The organized structure of the hippocampal formation allows electrophysiologists to stimulate a large number of excitatory fibers with a single stimulation electrode and record extracellularly from many neurons that are simultaneously activated. Typically, that results in a large inward current at the dendritic layer that could be detected and further analyzed. In addition, the possibility to retain the compact hippocampus structure, even in brain slices, led to a large accumulation of knowledge on synaptic transmission in the past few decades, via the vast contribution of *in vitro* electrophysiological studies.

The *in vivo* electrophysiological characteristics of the direct and indirect inputs to the hippocampal CA1 region have been studied in many different ways. Ironically, the tri-synaptic input has been successfully implicated by monosynaptic activation of the Schaffer collaterals to the CA1-sr *in vivo* (Manahan-Vaughan, 1997; Leung et al., 2003; Kemp and Manahan-Vaughan, 2004; Kemp and Manahan-Vaughan, 2008). On the other hand, the direct input from the EC to the CA1 region has been studied *in vivo* by the stimulation of the perforant path that inevitably activates the DG and subsequently the CA3 that results in mono-synaptic (EC-CA1) as well as di-synaptic (EC-CA3-CA1) and tri-synaptic (EC-DG-CA3-CA1) responses at the recording site (Yeckel and Berger, 1990, 1995).

The SC-CA1 synapse is typically represented by a negative-going single fEPSP at the CA1-sr that is comparably well characterized electrophysiologically. It has a short latency (<2 ms) due to the closeness of the Schaffer collaterals to the recording site and a rising phase in the range of 4-5 ms that is reflecting the large population of activated dendrites. On the other hand, the stimulation of the angular bundle *in vivo* reveals a start of fEPSP with a comparably longer latency and shorter rising phase that suits with the notion that the stimulation site is farther away and the stimulation is limited in terms of the number of contacts as would be expected in a topographically organized input relationship.
Typically for the Sc-CA1 synapse, increasing the stimulation intensity recruits larger number of afferent fibers that is measurable as the increase in the slope and the amplitude of the resulting fEPSP. A similar pattern of input-output relationship has been observed in the present study for both the Schaffer collateral and direct cortical input to the CA1. However it is difficult to have a clear resolution in vivo, since the contamination of the pp-CA1 synapse by the volume-conducted currents from the DG, as well as the involvement of the trisynaptic path activation is inevitable (Stringer and Colbert, 1994; Buzsaki et al., 1995). Thus, an increased intensity of stimulation would presumably result in a larger number of perforant path fibers recruited not only for the CA1 region but also for the DG and CA3 regions. As a consequence of this, the evoked potentials recorded from the distal dendrites of the CA1 region bear the appearance of a positive going sharp inflection (possibly volume conducted from the DG; Stringer and Colbert, 1994) as well as a long latency (>7 ms) negative going fEPSP (possibly due to the massive activation of the trisynaptic route; Leung et al., 1995). Recent findings however point to a reentrant pathway from the EC layer III to CA1-slm that would be represented via a second, late latency negative going fEPSP at the distal dendrites of the CA1 (Kloosterman et al., 2004; Bartesaghi and Gessi, 2003).

The input-output relationship of a particular synapse is important for determining the appropriate stimulus intensity to carry out research on synaptic plasticity. It has been previously shown that a low level of stimulus intensity enhances the fEPSPs more than a high intensity stimulus (Cao and Leung, 1991; Leung and Au, 1994; Jeffery, 1995; Foster and Dumas, 2001). Typically the stimulus intensity used to study basal synaptic transmission and plasticity in vivo is around the value that elicits 40% of the maximum measure of interest (slope or amplitude of the fEPSP) to avoid saturation of the synapses and allow standardization among different subjects as well as different laboratories (Leung and Au, 1994; Jeffery, 1995). For the Sc-CA1 synapse the input-output relationship elicits a sigmoidal curve that was also observed for the pp-CA1 synapse. Typically, the synapse is excited due to high-intensity stimulation and requires a period to recover to the initial excitability level. A period of thirty minutes has been used in our laboratory successfully in a large number of studies (Manahan-Vaughan, 2000; Kemp and Manahan-Vaughan, 2004, 2007a). However, the pp-CA1 synapse appeared to require longer period of time to return to the basal levels of excitation (Fig. 3.7). The lowest intensity of stimulation elicited approximately 2-fold of the
initial response even two hours after the accomplishment of the initial input-output profile. Technically, it becomes almost impossible to determine a normalized stimulus intensity in a short period of time using the initial input-output profile.

Functionally, it is extremely difficult to judge about the nature of this phenomenon. The main constraint is the possible volume conduction due to the massive activation of the granule cells of the dentate gyrus that is not avoidable in the intact brain (Stringer and Colbert, 1994; Buzsaki et al., 1995). However, if allowed to make a suggestion it is possible that a lack of homeostatic regulation (reviewed in Turrigano, 2007) is observed after the brief high-intensity stimulation during the establishment of the input-output curve.

4.2.3 Ultra short term plasticity at pp-CA1 and Sc-CA1 synapses

Ultrashort-term plasticity is generally accepted to be a reflection of presynaptic characteristics for a given synapse, but do not exclude a possible post-synaptic contribution (Dobrunz and Stevens, 1999; Thomson 2000; Zucker and Regehr, 2002). Ultrashort-term plasticity can be studied in terms of paired pulse administration with a range of interpulse intervals that vary from several milliseconds to seconds that result either in facilitation or depression of the second response compared to the first one (reviewed in Zucker and Regehr, 2002). The degree of paired-pulse facilitation or depression has been shown to be reliably related to the probability of transmitter release, the number of release sites as well as the quantal amplitude among other pre- and post-synaptic characteristics (for detailed review see Thomson, 2000; Zucker and Regehr, 2002). Paired-pulse examination has also been shown to carry information about the inhibitory circuits in the hippocampus (Bronzino et al., 1996, 1997; Miles et al., 1996; Davies et al., 1991).

Paired-pulse stimulation of the hippocampal synapses has been successfully applied both in vitro (Speed and Dobrunz, 2009) and in vivo (under anesthesia (Maruki et al., 2001; Leung et al., 1995) and in freely behaving animals (Austin et al., 1989; Bronzino et al., 1997; Madronal et al., 2009; Cao and Leung, 1991) for the Sc-CA1 synapse as well as pp-DG synapse. Regarding the pp-CA1synapse limited evidence is present from in vitro cases (Speed and Dobrunz, 2009) and from animals under anesthesia (Maruki et al., 2001). There exists no study performed under freely behaving conditions to our knowledge. Although the
methodology differs, the results from this work are consistent with the ones obtained by Leung et al. (1995). They have shown that the current sink at the CA1-slm studied by current-source density analysis under urethane anesthesia is facilitated via paired pulse stimulation with 50 ms interpulse interval. Paired-pulse facilitation occurs at the pp-CA1 synapse in vitro as well (Speed and Dobrunz, 2009). The general curve characteristics in vitro and the ones from this work are not consistent, here we did observe comparably moderate paired-pulse facilitation for the intervals below 100 ms that peak around 40 ms interval. Apart for the fact that the intact brain would have a preserved anatomical network, several other factors could account for that discrepancy, among which are the block of the GABAergic inhibition (via picrotoxin) and NMDA receptors (via AP5) in vitro (Speed and Dobrunz, 2009) but not in vivo. Regardless of the methodological differences, the overall interpretation could be that the findings overlap in the sense that the paired-pulse responses of the Sc-CA1 and pp-CA1 synapses are similar for the young adult rats with moderate or low intensity of stimulation.

The intensity of stimulation appears to be responsible for the direction of the paired-pulse response towards facilitation or depression for low and high stimulus intensities, respectively (Madronal et al., 2009). It has been shown in freely behaving mice that progressively increasing the stimulation intensity shifts the paired-pulse facilitation to paired-pulse depression at the Sc-CA1 synapse (Madronal et al., 2009). Consistent with their results, it was observed in the present work that paired-pulse depression significantly dominates at high-intensity stimulations of the Sc-CA1 synapse. However, the pp-CA1 synapse responses were not in line with those from the Sc-CA1, rather the paired-pulse ratio curves for the pp-CA1 animals had similar pattern regardless of the stimulation intensity. Given that the paired-pulse facilitation has been inversely correlated with the pre-synaptic release probability, the results of this work are also consistent with the fact that perforant path terminals at the CA1 indeed have lower release probability compared to the Schaffer collaterals (Ahmed and Siegelbaum, 2009). Another possible explanation might be the activity of the pre-synaptic group II mGluR receptors at the pp-CA1 synapse that would further limit the release probability upon activation (Kew et al., 2001) or presynaptic dopaminergic effect as previously shown (Otmakhova and Lisman, 1999, 2000). The contribution of the post-synaptic mechanisms to the paired-pulse ratio profiles obtained in this work is also likely since the postsynaptic
molecular composition of the CA1-slm and the CA1-sr has been shown to be dissimilar (Tsay et al., 2007; Komendantov and Ascoli, 2009).

Another aspect of the paired-pulse responses is the possible visualization of the fast inhibitory effect on the cell bodies with short interpulse intervals (typically < 40ms) that has been observed both in vivo and in vitro for Sc-CA1 as well as pp-DG connections (Bronzino et al., 1997). When the stimulation intensity is strong enough to evoke a population spike at the cell layer, fast GABAergic activation prevents the appearance of population spike at the subsequent response (Thomson, 2000; Zucker and Regehr, 2002). Consequently, assuming that the evoked responses at the pp-CA1 synapse innately comprise volume-conducted currents travelling from the granule cell layer of the DG, short-interval paired pulses should be capable of eliminating the positive going inflections superimposed on the early fEPSP at the pp-CA1 synapse. However, the results from this work are not in line with this interpretation. The possibility exists that with low-intensity stimulation, the generation of the population spike at the DG granule cells fails, and the population spike in the second response is generated (Bronzino et al., 1997). On the other hand, it would be unlikely that the same explanation holds for high-intensity stimulation that should typically be strong enough to generate population spikes for the pp-DG connection. Indeed in the present work it has been observed that the appearance of the positive going inflection on the pp-CA1 fEPSP is more consistent with high-intensity stimulation (as would be expected from the input-output relationship examined in advance) and it is not attenuated but conversely facilitated. Previously, the behavioral state of the animal has been shown to interfere with the paired-pulse ratio of the pop-spike (Cao and Leung, 1991). Although all the recordings were performed when the animals were awake and sitting still, whether the results of this work were affected by the behavioral state of the animal, or not, is unclear.

Although it provides insight about the characteristics of the synapses, the interpretation of the ultrashort-term plasticity in terms of naturally occurring phenomena in the brain is rather difficult. The general understanding is that the short-term plasticity (including ultrashort-term plasticity) serves temporal information processing and modulation of transient responses (reviewed in Lisman, 1997; O’Donovan and Rinzel, 1997; Fisher et al., 1997; Fortune and Rose, 2001).
4.2.4 Long term synaptic plasticity at the pp-CA1 synapse

Long term persistent synaptic plasticity induction at the pp-CA1 synapse has led to a debate, since the direct input to the CA1 has been shown to result only in a weak excitatory and a strong inhibitory activation (Colbert and Levy, 1992; Buzsaki et al., 1995; Levy et al., 1995; Soltezs, 1995; Soltezs and Jones, 1995). Recent studies, however, reported the successful induction of long-term persistent synaptic plasticity at the pp-CA1 synapse in vitro (Remondes and Schuman, 2002, 2003, 2004; Dvorak-Carbone and Schuman, 1999a, 1999b).

The induction of the LTP at the pp-CA1 synapse has been shown to have contradictory results in vitro (Colbert and Levy, 1992, 1993; Dvorak-Carbone and Schuman, 1999a, Remondes and Schuman, 2002, 2003). Typical stimulation with high frequency was thought to activate the inhibitory transmission in the first attempts to study LTP at the pp-CA1 synapse (Colbert and Levy, 1992). Whereas, later attempts successfully elicited LTP either with HFS or theta burst stimulation in the presence of intact inhibitory transmission (Dvorak-Carbone and Schuman, 1999; Remondes and Schuman, 2002, 2003).

The present work describes long-term persistent potentiation at the pp-CA1 synapse in freely behaving rats. At other hippocampal synapses such as the Sc-CA1 synapse or the pp-DG synapse, strong high-frequency stimulation (HFS) induces LTP (persistent, longer than 24h at least) whereas weaker HFS induces decremental LTP that turns to basal synaptic values within few hours (short-term potentiation, STP) (Kemp and Manahan-Vaughan, 2004, Frey and Frey 2009). Thus, to examine synaptic plasticity at the pp-CA1 synapse four different HFS protocols, of differing frequency and differing intensity were studied.

HFS at 100 Hz results in a well characterized LTP at the Sc-CA1 synapse (Kemp and Manahan-Vaughan, 2004). The results of the present work reveal a similar profile of potentiation at the pp-CA1 synapse compared to Sc-CA1 synapse using the same HFS protocol. Expectedly, a stronger stimulus (100 Hz in four as opposed to one burst) had a more robust effect compared to the weak stimulus.

In addition, weak (three bursts) and strong (ten bursts) HFS at 200Hz were tested for their reliability in inducing STP and LTP, respectively in studies of the perforant path-DG synapse, (Manahan-Vaughan et al., 2000; Kemp and Manahan-Vaughan, 2008; Frey and Frey 2009).
Here, however, we consistently observed LTP, the magnitude of which depended on the strength of stimulus. Interestingly, enhancement of the fEPSPs at the pp-CA1 synapse did not return to basal levels even 24 h after weak HFS, suggesting that the pp-CA1 synapse is capable of expressing robust and very persistent LTP.

The molecular basis of LTP induction at the Sc-CA1 and pp-CA1 synapses is thought to differ. The backpropagation of action potentials fulfills the Hebbian criterium of presynaptic-postsynaptic activation coupling for strengthening (or weakening) of the Sc-CA1 synapse (Magee and Johnston, 1997; Mehta, 2004; Nevian and Sakmann, 2006). Nonetheless, backpropagation of action potentials may not be a sufficient trigger for LTP at the distal apical dendrites, even at the CA1-sr layer, as the action potentials attenuate dramatically with increasing distance from the soma (Spruston et al., 1995; Andreasen and Lambert 1995; Stuart et al., 1997). In contrast, stimulation of the perforant path is not sufficient to evoke action potentials at CA1 neurons (Colbert and Levy, 1992; Buzsaki et al., 1995; Soltezs, 1995; Soltezs and Jones, 1995). Induction of LTP at the distal synapses of the CA1 region appears to necessitate simultaneous activation of afferents, but not the back propagation of the somatic action potentials (Golding et al., 2002; Hardie and Spruston, 2009). In fact, local dendritic spikes, that allow large calcium influx, are thought to be the major contributors for LTP induction at the distal synapses. This in turn depends on combined functioning of NMDA receptors and voltage-gated calcium channels (Golding et al., 2002; Gasparini et al., 2004; Mehta, 2004).

Low-frequency stimulation (LFS) at 1 Hz (900 times to elicit LTD, or 600 times to elicit STD) has been shown to be an extremely reliable protocol for induction of synaptic depression at the Sc-CA1 synapse in freely behaving rats (Manahan-Vaughan, 2000).

LFS of the perforant path (1 Hz stimulation for 10 or 15 minutes) results in homosynaptic LTD of pp-CA1 synapse in vitro (Dvorak-Carbone and Schuman, 1999b; Wohrl et al., 2007) that persists for longer than an hour, can be saturated by repeated application of the stimulation protocol and reversed by the application of HFS (Dvorak-Carbone and Schuman, 1999b). Furthermore, LTD induced at the pp-CA1 synapse is not affected by a block of inhibitory activity of GABAergic receptors in one study (Dvorak-Carbone and Schuman, 1999b) but requires slow inhibitory transmission exerted via GABA-B receptors in another
Discussion

(Wohrl et al., 2007). The variations on the results could be due to methodological differences and the difficulty of preventing contamination from the potent Schaffer collaterals even in vitro, while preserving a large amount of intact entorhinal cortex fibers (Dvorak-Carbone and Schuman, 1999b; Wohrl et al., 2007).

Dvorak-Carbone and Schuman, in their in vitro study (1999b), showed that LTD at the pp-CA1 synapse can be induced even with a protocol that is not strong enough to affect the Sc-CA1 synapse. In the present work, however, application of LFS that is known to induce short-term depression (STD) at the Sc-CA1 synapse in freely behaving animals (Manahan-Vaughan, 2000) failed to induce any change in the basal synaptic transmission at the pp-CA1 synapse under similar conditions.

One of the effective factors in induction of long-term plasticity is the intensity of stimulation (Leung and Au, 1994; Jeffery, 1995). Thus, the intensity of stimulation during LFS was increased to evoke responses that reflect fEPSPs that comprise 70% of the maximum obtained in the input-output curve. LFS given at this intensity evokes persistent LTD in Sc-CA1 synapses (Manahan-Vaughan and Braunewell, 1999, 2005; Kemp and Manahan-Vaughan, 2004, 2005, 2008a, 2008b; Lemon and Manahan-Vaughan, 2006; Lemon et al., 2009) as well as mossy fibers to CA3 (Hagena and Manahan-Vaughan, 2010; Klausnitzer and Manahan-Vaughan, 2008) synapses.

Interestingly, increasing the stimulation intensity during the LFS resulted in synaptic potentiation (< 3h) at the pp-CA1 synapse in freely behaving animals. The simplest explanation for this surprising outcome is interference derived from the increased excitability and lack of flexibility of the pp-CA1 synapse, in relationship to the increased intensity of stimulation. A positive-going inflection appeared in the fEPSP after the LFS was given, thus complicates the overall picture, as it suggests that volume-conduction from the activated DG contaminated the fEPSP (Leung, 1995; Stringer and Colbert, 1994; Buzsaki, 1995). Nonetheless, the possible indirect effects of volume conduction from the Sc-CA1 and pp-DG activation do not explain the potentiation in its entirety since both the Sc-CA1 and pp-DG synapses undergo LTD following LFS at 1 Hz (and even at a similar stimulation intensity as used here) (Manahan-Vaughan and Braunewell, 1999, 2005; Kemp and Manahan-Vaughan, 2004, 2005, 2008a, 2008b; Lemon and Manahan-Vaughan, 2006; Lemon et al., 2009).
At other synapses, LFS (1Hz) does not always produce synaptic depression (Habib and Dringenberg, 2010). In fact, 1 Hz LFS of excitatory afferents to the basolateral amygdala in vitro elicits synaptic potentiation that persists for at least thirty minutes (Li et al., 1998, 2001). Follow-up in vitro research by Huang and Kandel (2007) confirmed LFS-induced LTP at cortico-amygdala synapses that is pathway specific and regulated by neuromodulators (e.g., dopamine, serotonin). However, to extrapolate those phenomena to hippocampal synapses might be erroneous as it is known that long-term synaptic plasticity is not a unitary mechanism and differs in terms of induction as well as maintenance requirements among different synapses even for the hippocampal subregions (Kemp and Manahan-Vaughan, 2004, 2007a, 2008; Hagen and Manahan-Vaughan, 2010). Evidence exists, however, that gradually evolving LTP could be induced at the Sc-CA1 synapse if the LFS (1Hz) is applied for short periods (3-5 min) (Lante et al., 2006a, 2006b). Furthermore, a recent study suggests distance dependent differential effects of LFS on the CA1 apical dendrites that span the CA1-sr layer (Parvez et al., 2010).

What could be the reason why the pp-CA1 synapse has a different response to a typical LFS compared to other hippocampal inputs (pp-DG, Sc-CA1)? One possible explanation resides in the anatomical specificity of the EC-hippocampal connections (discussed in detail in section 4.2.1). The superficial layers II and III of the entorhinal cortex not only give rise differentially to the tri-synaptic and monosynaptic CA1 inputs, respectively, but they also have different electrophysiological characteristics (Gloveli et al., 1997a, 1997b, 1997c; van der Linden and Lopes da Silva, 1998). Interestingly, layer III neurons, that are the origin of the pp-CA1 synapse, generate action potentials preferentially in response to LFS (< 10 Hz) compared to the HFS (10-40Hz) activated layer II neurons (Gloveli et al., 1997b). Assuming that angular bundle stimulation activates EC layer III neurons (as was previously shown in vitro, Naber et al., 2001; Yoshida et al., 2008), persistent LFS might actually favor the activation of the layer III neurons and reveal the potentiation effect, instead of the expected depression effect that occurs at the terminals of the layer II projection (mainly DG and CA3). Such an explanation corroborates with the findings of Yeckel and Berger (1995), who showed that the monosynaptic excitation of the hippocampal sub-regions is superior following LFS (< 5 Hz), compared to the multisynaptic excitation (that is favored for frequencies higher than 10 Hz) in intact rabbit brain. Similarly, Bartesaghi and Gessi (2003) observed activation in EC layer III
and subsequently showed a current sink in the CA1-slm in response to repetitive (> 5 pulses) LFS (1.0 – 1.5 Hz) in guinea pig brain in vivo (but see Kloosterman et al., 2004). However, no study exists (to the author’s knowledge) that examines the response of the CA1-slm to persistent angular bundle stimulation with LFS in vivo. The common point within the aforementioned studies and the current one, is that the EC is preserved and the angular bundle actually contains not only the EC (layer II, III) input to the hippocampal formation but also the hippocampal output that innervates the deep layers of EC (Tamamaki and Nojyo, 1995). Whereas, an in vitro experimental set-up using the stimulation site close to CA1-slm in an isolated patch of CA1, will not involve activation of the layer III EC (Dvorak-Carbone and Schuman, 1999b; Wohrl et al., 2007). Thus, a possibility is that the unusual outcome of the LFS stimulation of the angular bundle is related to the origin of the input.

The methodology used in this work does not provide direct evidence for the nature of the multisynaptic effect at the recording site that most probably is innately present due to the intact hippocampal network for the in vivo preparations compared to in vitro studies (discussed in detail in section 4.2.1). Thus, another possible explanation for the LFS-induced potentiation at the pp-CA1 synapse involves the inhibitory network that affects the outcome of the pp-CA1 synapse (Blasco-Ibanez and Freund, 1995; Freund and Buzsaki, 1996; Dvorak-Carbone and Schuman, 1999b; Klausberger, 2008, 2009). Accordingly, CA1-slm is thought to be a target for both feed-forward and feed-back inhibition (reviewed in Freund and Buzsaki, 1996; Pelletier and Lacaille, 2008; Klausberger, 2009). Interesting in relation to this work, are the findings of Maccaliferri and McBain (1995) that elegantly demonstrate the effect of LTD induction at the Sc-CA1 synapse on the oriens-lacunosum moleculare interneurons that further regulates the activity of the pp-CA1 synapse. In their study Maccaliferri and McBain (1995) show that LTD at the Sc-CA1 synapse is passively transferred to the oriens-lacunosum moleculare interneurons and leads to disinhibition of the pp-CA1 synapse presented as potentiation of the evoked responses from the perforant path test pulses (Maccaliferri and McBain, 1995). Recently, a similar heterosynaptic relationship was shown for the CA1-subiculum synapse, where LFS (1Hz, 15min) application to the pp-CA1 synapse results in heterosynaptic disinhibition at the CA1-subiculum synapse in vitro (Fidzinski et al., 2010). However, both of the explanations above involve specific multisynaptic activation that is not directly addressed in the present work and remain to be explored in further studies.
What might be the functional significance of LFS-induced potentiation at the pp-CA1 synapse and does it actually occur under physiological conditions? Indeed, slow oscillations with a frequency of around 1 Hz were implicated as a state in slow-wave-sleep that show a current sink in the CA1-slm layer (Wolansky et al., 2006). Interestingly, the response of the pp-CA1 synapse during the spontaneously occurring slow oscillations appears amplified whereas the pp-DG synapse has a reduced response (Schall and Dickson, 2010). Thus, it is quite possible that the consolidation of memories during sleep is reinforced by slow oscillations in different sub-regions of the hippocampus, as proposed previously (Brun et al., 2002; Remondes and Schuman, 2004).

4.2.5 Regulation of synaptic plasticity at CA1 synapses by NMDA receptors and L-type voltage-gated calcium channels

Induction of persistent synaptic plasticity requires postsynaptic calcium ion entry via the activation of NMDA receptors in many different synapses in the brain (Malenka and Nicoll, 1999; Morgan and teyler, 1999). It was shown by our group and others that LTP processes at the Sc-CA1 and pp-DG synapses necessitate activation of NMDA receptors in vivo (Manahan-Vaughan et al., 1998; Fox et al., 2006). However, NMDA-independent LTP also occurs that necessitates activation of voltage-gated calcium channels (VGCC) (Grover and Teyler, 1990). The combined NMDA/VGCC involvement in LTP was shown for both 200Hz-induced HFS in Sc-CA1 synapses (Freir and Herron, 2003) and 400Hz-induced HFS in pp-DG synapses (Manahan-Vaughan et al., 1998).

The HFS protocol to test the involvement of NMDA receptors in LTP observed at the pp-CA1 synapse comprised 200Hz stimulation (3 bursts) because of the previously reported differential frequency-dependency of LTP at Sc-CA1 and pp-DG synapses (Manahan-Vaughan et al., 1998; Freir and Herron, 2003). Here, 100 Hz stimulation elicits LTP at Sc-CA1 synapses (Manahan-Vaughan, 1997), whereas this frequency only elicits STP at pp-DG synapses (Manahan-Vaughan, personal communication). In contrast, 200 Hz stimulation elicits LTP at pp-DG synapses (Manahan-Vaughan et al., 1998), whereas this frequency causes epileptiform seizures at SC-Ca1 synapses (Manahan-Vaughan, personal communication). As VGCCs are first recruited with 400 Hz HFS at pp-DG synapses (Manahan-Vaughan et al., 1998), it was presumed that 100 Hz HFS at pp-CA1 synapses
would have been insufficient to recruit VGCCs in the LTP process. In the present work, blocking NMDA receptors via D-AP5 significantly reduced the amplitude of the LTP (wHFS at 200 Hz) at the pp-CA1 synapse but failed to abolish it completely. These results suggest that LTP (200Hz) at the pp-CA1 synapse involves calcium influx by additional means other than NMDA receptors in vivo similar to what was implicated in the in vitro reports (Remondes and Schuman, 2002, 2003; Golding et al., 2002).

Previously, Remondes and Schuman (2002, 2003) reported a partial involvement of the NMDA receptors in HFS- or theta-burst induced LTP at the pp-CA1 synapse. The residual LTP capacity of the pp-CA1 synapse after the block of NMDA receptors appears to be dependent on VGCCs (Remondes and Schuman, 2002; 2003; Golding et al., 2002; Mehta, 2004). Thus, to test if the same mechanism applies in intact brain, a combination of NMDA receptor and L-type VGCC block was employed. The results are in line with the abovementioned in vitro findings (Remondes and Schuman, 2002, 2003), although a small potentiation was still present afterwards. It is unlikely that the concentration of the drugs used was insufficient to block the NMDA receptors and VGCCs, as it was previously shown that higher concentrations of the NMDA receptor antagonist D-AP5 or the VGCC-blocker does not further affect the LTP expression (at least not for the 400Hz induced LTP at the pp-DG synapse) in freely behaving rats (Manahan-Vaughan et al., 1998). However, the higher NMDA/AMPA receptor contribution at the pp-CA1 synapse (Otmakhova and Lisman, 2002) might necessitate higher concentrations of D-AP5 to be tested in the future, in order to verify this assumption.

The remaining potentiation after the combined block of NMDA and L-type VGCCs might involve other receptor types such as Ni-sensitive R- and T- type VGCCs that are thought to contribute to the calcium influx at the distal CA1 dendrites (Magee et al., 1998; Isomura et al., 2002), metabotropic glutamate receptors, as shown previously for the pp-DG synapse in vivo (Manahan-Vaughan et al., 1998) or calcium permeable AMPA receptors (Yuste et al., 1999). In general, the 200Hz-HFS induced LTP at the pp-CA1 synapses is similar to LTP at the Sc-CA1 synapse, but not to the pp-DG synapse. Further research would be needed to clarify the exact molecular mechanisms.
4.2.6 Regulation of basal synaptic transmission at CA1 synapses by group II mGluRs

Group II mGluRs (mGluR2 and mGluR3) are G-protein coupled receptors that are densely located perisynaptically on the presynaptic terminals of the perforant path and on mossy fibers of the hippocampal formation (Shigemoto et al., 1997). Group II mGluRs are negatively coupled to the cAMP-protein kinase A signaling cascade and thought to act as autoreceptors modulating glutamate release (Cartmell and Schoepp, 2000). The activation is exerted not only on the principal neurons but also on interneurons and can be detected readily as a decrease in basal synaptic transmission (Manahan-Vaughan, 1997; Kulla et al., 1999; Hagena and Manahan-Vaughan, 2010; Speed and Dobrunz, 2009; Semyanov and Kullmann, 2000). However, under physiological conditions, group II mGluRs have an activity-dependent involvement that probably requires glutamate spill-over (Kulla et al., 1999; Kew et al., 2001; Manahan-Vaughan, 1997; Scanziani et al., 1997).

Previously, activation of group II mGluRs was shown to reduce basal synaptic transmission in the Sc-CA1, pp-DG and mossy fiber-CA3 synapses in a concentration-dependent manner in freely behaving rats (Manahan-Vaughan, 1997; Kulla et al., 1999; Hagena and Manahan-Vaughan, 2010). For example, the mossy fibre-CA3 synapse has a high sensitivity to the activation of group II mGluRs compared to the pp-DG synapse, a characteristic that is successfully used to screen the mossy fibre-CA3 synapse in vivo (Hagena and Manahan-Vaughan, 2010). A similar approach was used in vitro by Speed and Dobrunz (2009) to identify the highly sensitive pp-CA1 synapse from the Sc-CA1 synapse. The results of the present work further confirm the inhibitory effect of group II mGluR activation on the basal synaptic transmission at the pp-CA1 synapse in freely behaving animals. The effect was observed shortly after the drug application and persisted for about 2h. The concentration of the group II mGluR agonist, DCG-IV, used in the present work is higher compared to the concentration that is effective in inhibiting basal synaptic transmission at the mossy fiber-CA3 synapse implying a lower sensitivity of the pp-CA1 synapse. Nonetheless, the results are in line with in vitro reports that show a comparable DCG-IV sensitivity profile for the pp-CA1 and pp-DG synapses (Kew et al., 2001).

In general, application of group II mGluR antagonists reverses the effect of group II mGluR agonists (Manahan-Vaughan, 1997; Kew 2001; Giacomo and Hasselmo, 2006) but fails to
induce changes in basal synaptic transmission (Manahan-Vaughan, 1997; Kew et al., 2001; Giacomo and Hasselmo, 2006) that acts as an indicator of activity-dependence under physiological conditions (Kew et al., 2001). In fact, application of the group II mGluR antagonist, EGLU, affects LTD but not LTP in the Sc-CA1 and pp-DG synapses in freely behaving rats and has no effect on basal synaptic transmission (Manahan-Vaughan, 1997; Altinbilek and Manahan-Vaughan, 2007). For the pp-CA1 synapse, Kew et al (2001) has shown that antagonism of group II mGluRs inhibits the auto-inhibitory effect of the receptors after burst stimulation, but does not contribute to basal synaptic transmission. In the present study however, one was surprised to find that basal synaptic transmission at the pp-CA1 synapse was suppressed following group II mGluR antagonism.

A possible explanation for this effect might be the differential response of the inhibitory and excitatory networks to the modulatory action of mGluRs. Activation of group II mGluRs has a depressive effect on basal synaptic transmission of the interneurons located at the stratum lacunosum moleculare similar to the effect observed at the pp-CA1 synapse, whereas antagonism does not have impact (Price et al., 2005). Thus, the overall effect might be a competition between the excitatory and the inhibitory network that would account for lower sensitivity of the pp-CA1 synapse to DCG-IV. On the other hand, Losonczy et al. (2003) observed that interneuron types respond differentially to the activation/block of group II mGluRs. Interestingly, the specific oriens-lacunosum moleculare interneurons that terminate in the CA1-slm (Klausberger, 2009) show an increased response in the presence of a group II mGluR antagonist and are not drastically affected by the application of DCG-IV (Losonczy et al., 2003). The methodology used in this work does not allow to distinguish the excitatory network from the inhibitory network, however in light of previous reports the overall effect of EGLU might be represented as increased inhibition at the pp-CA1 synapse.

4.2.7 Synaptic plasticity at the pp-CA1 synapse where the CA3 is severed

Whether the role of the direct input on the CA1 is independent or only modulatory to the trisynaptic circuit was another challenge to address. In vitro experiments suggested a potent modulatory role for the perforant path input, exerted on synaptic transmission and synaptic
plasticity of the Sc-CA1 synapse (Levy et al., 1998; Dvorak-Carbone and Schuman, 1999a; Remondes and Schuman, 2002; Migliore, 2003; Judge and Hasselmo, 2004; Ang et al., 2005; Dudman et al., 2007; Wohrl et al., 2007; Izumi and Zorumski, 2008). LFS of the perforant path resulted in either transient depression (<5min) (Izumi and Zorumski, 2008) or no effect (Dvorak-Carbone and Schuman, 1999b) on the Schaffer collateral evoked responses. Heterosynaptic LTP of the Sc-CA1 pathway occurs when LFS is given to the perforant path (Wohrl et al., 2007). On the other hand, homosynaptic LTP induced by stimulation of the Schaffer collaterals is reliably depotentiated by persistent low frequency stimulation (1Hz 900 pulses) of the perforant path (Izumi and Zorumski, 2008). The depotentiation appears to be potent and long lasting and relies specifically on the stimulation of the perforant path, as it is not observed by stimulation of a second Schaffer collateral input (Izumi and Zorumski, 2008). Another important feature is that it does not occlude the further induction of LTP at the Sc-CA1 synapse that would increase the resistance to saturation of the synapse and enlarge the functional window (Izumi and Zorumski, 2008).

To isolate the pp-CA1 synapse and examine the effect of the tri-synaptic input, a stainless steel cannula or a thin plate was chronically implanted at the Schaffer collaterals, therefore partially severing the CA3 from the CA1 at the dorsal part of the hippocampus. Cutting the Schaffer collateral fibers mechanically is not a unique approach for this work. Previously Brun et al. (2002) shown that it is possible to dissociate the CA3 input to the CA1 by introducing minirazor blade cuts at the Schaffer collateral fibers along the septo-temporal axis of the hippocampus and retain the perforant path input functional. The cut used in this work is comparably limited to the anterior parts of the hippocampus and spares the contralateral contribution of the CA3 region, however appears to be sufficient enough to destroy the input to the recording site.

The animals with severed CA3 did not differ significantly from the intact group when HFS protocols were applied, suggesting no interference of the Schaffer collateral input to the LTP induction at the pp-CA1 synapse. Interestingly, the LFS protocol (1Hz, 10 min; 70%) that potentiated the pp-CA1 synapse in intact animals resulted in LTD when the CA3 was severed. The induction of LTD is not attributable to a non-specific brain damage since induction of LTD is an active process that requires anatomical as well as molecular integrity of the
synapse (Mulkey and Malenka, 1992; Bear and Abraham, 1996; Hrabetova and Sacktor, 1997).

The successful induction of LTD where the Sc input is severed, is well in line with in vitro findings where the CA3 is separated from CA1 for technical reasons (Dvorak-Carbone and Schuman, 1999b). These results also favor the possibility that the Sc-CA1 synapse is actively corresponding to the outcome of LFS at the pp-CA1 synapse. This may possibly occur through the activation of the feed-back inhibitory system (Maccaferri and McBain, 1995) (discussed in detail in section 4.2.4).

Another conclusion is that induction of LTP at the pp-CA1 synapse with different HFS frequencies is not affected by the Schaffer collateral input, suggesting differential network activity of the hippocampal sub-regions to LTP and LTD protocols.

4.2.8 Learning-facilitated plasticity at the pp-CA1 synapse

It was previously shown in our laboratory that electrically-induced short-term plasticity, lasting hours, is facilitated to long-term plasticity that persists for days, if a spatial learning experience occurs when patterned afferent stimulation (to induce short-term plasticity) is given (Manahan-Vaughan and Braunewell, 1999; Kemp and Manahan-Vaughan, 2004, 2007a, 2008). For example, a general change in the environment significantly prolongs the duration of electrically-induced potentiation at the Sc-CA1 and pp-DG synapses (Kemp and Manahan-Vaughan, 2004, 2007a, 2008). The results of the present study however, did not link the exposure to a novel environment with the plasticity at the pp-CA1 synapse as there was neither a facilitatory nor an inhibitory effect compared to LTP induced by HFS alone. However, the technical difficulties in studying the pp-CA1 synapse in vivo should be not forgotten (discussed in section 4.2.1). The effect of volume-conduction due to the simultaneous activation of DG, and subsequently the Sc-CA1 synapse, possibly interferes with the outcome at the pp-CA1 synapse. Assuming the same degree of learning-facilitated potentiation simultaneously occurs at both of the synapses, anatomically surrounding the CA1-slm, the net effect of volume conduction might either cancel the changes at the pp-CA1 synapse or at the very least, mask changes in synaptic strength resulting in ostensibly neutral results.
Learning-facilitated plasticity has also been studied in terms of the effect of changes in the context of the environment. Exploration of small, partially concealed objects within the holes of the holeboard facilitates LFS-induced LTD (Manahan-Vaughan and Braunewell, 1999; Kemp and Manahan-Vaughan, 2004) and depotentiates the HFS-induced LTP (Manahan-Vaughan and Braunewell, 1999; Kemp and Manahan-Vaughan, 2004) at the Sc-CA1 synapse. On the other hand, the pp-DG synapse does not respond with facilitated plasticity to these stimuli (Kemp and Manahan-Vaughan, 2008). Rather, large environmental cues facilitate LTD at the pp-DG synapse (Kemp and Manahan-Vaughan, 2008). For the pp-CA1 synapse, the exploration of fine spatial cues during HFS application facilitated both the early and the late phase of the electrically-induced LTP. These results are consistent with the notion that the CA1 region is sensitive to the changes of the environment in the form of small contextual cues. As the depotentiation caused by object exploration at the Sc-CA1 synapse shares a common mechanism with LTD induction (Kemp and Manahan-Vaughan, 2004), the possible explanation for the results of this study could be that the procedures inducing LTD at the Sc-CA1 synapse disinhibit the potentials at the pp-CA1 synapse or lead to a potentiation (discussed also in section 4.2.4). Still the opposite interaction is not ruled out and the pp-CA1 synapse might have a role in the expression of LTD at the Sc-CA1 synapse. The volume-conduction effect from the DG is rather difficult to predict as direct evidence exists that under the same circumstances used in this study no facilitation of plasticity occurs at the pp-DG synapse (Kemp and Manahan-Vaughan, 2008).

In animals that had undergone severance of the CA3 input to CA1, LFS was given whilst the animals explored the novel environment. These animals express LTD when LFS is given alone. Under the conditions of spatial exploration, LFS-induced LTD was impaired. Thus, independent of the contribution of Schaffer collateral input, the learning-mediated, facilitatory effect of the exposure to small, partially concealed objects might share mechanisms with LTP at the pp-CA1 synapse, rather than LTD as in Sc-CA1 synapse.

The overall interpretation suggests that differential molecular mechanisms are employed for the information processing at the Sc-CA1 and pp-CA1 synapses. The sensory information conveyed directly from the EC layer III to the CA1 might prevalently contain details of the novel spatial context, whereas the indirect pathway might be involved in a more general interpretation of the spatial changes in the environment.
Discussion

Synthesis

The contribution of the pp-CA1 synapse to formation of long-term memories is believed to be as important as the Sc-CA1 synapse. Although direct experimental evidence is limited, the role of the direct cortical input has been considered in many learning and memory models (Rolls, 1996; Lisman, 2005; Rolls and Kesner, 2006; Jensen and Lisman, 2005; Treves, 2004; McClellan et al., 1995; Hasselmo and Eichenbaum, 2005).

Since the CA1 region is the only output from the hippocampus proper it is proposed to comprise the final matching module (mismatch detector and information integrator) of information arising from both the CA3 region and the direct sensory information from entorhinal cortex.

Recent studies point to the differential activation of cell assemblies in CA3 and CA1 region in response to change in environmental stimuli. It has been shown that a distinct set of pyramidal cells are activated in CA3 after a drastic change of environment, whereas in CA1 the activated populations overlap in terms of immediate early gene expression (Vazdarjanova and Guzowski, 2004) and firing pattern (Leutgeb et al., 2004). A time-difference occurs in development of the ensemble activity that comprises around 10 min for CA1 and 20-30 min for CA3 (Leutgeb et al., 2004). On the other hand, Lee et al. (2004) reported that place cells in the CA3 region respond to new cue configurations in the first exposure whereas in the CA1 region the difference takes a day to develop. In addition, place cell behaviour in the CA3 region appears to be more coherent compared to the CA1, in original and cue-altered environments (Lee at al., 2004). A more recent study also indicates the distinct, yet complementary, functioning of CA1 and CA3 in terms of storing ability of environmental cues. The cell assembles that respond to specific spatial locations appear to be active in the positive phase of theta cycle in CA3 and in the negative phase in CA1 (Dragoi and Buzsaki, 2006). Thus, the two hippocampal sub-regions, CA1 and CA3 are thought to work independently to some extent, which might be determined by the complexity and the specific requirements of the task to be acquired (Guzowski et al., 2004).
4.3 Concluding comments and new directions

The segregation of the tri-synaptic and the monosynaptic innervations on the CA1 region at gross anatomical as well as fine molecular level suggests differential mechanisms for synaptic plasticity induction that might contribute to the functional properties of the two inputs.

The direct pathway from EC layer III to the hippocampal CA1 region is challenging to address in freely behaving rats. Although the indirect contribution of the EC layer II input is not possible to ignore, several characteristics of synaptic plasticity are expressed differently at the pp-CA1 synapse compared to pp-DG and Sc-CA1 synapses.

The molecular mechanisms underlying the differences between the pp-CA1 and Sc-CA1 synapses in response to ultrashort-term plasticity, induced with high-intensity inputs, would be interesting to study for a better understanding of the presynaptic characteristics of the pp-CA1 synapse in vivo.

To what extent is the inhibitory network involved in the LFS-induced potentiation at the pp-CA1 synapse? It would be interesting to explore that finding pharmacologically, as it appears to be mechanically affected by the Schaffer collateral input.

In the framework of this work, group II mGluRs affected basal synaptic transmission at the pp-CA1 synapse in an unusual way compared to other hippocampal inputs. How the mGluRs, not only group II but also group I and III, contribute to the long-term persistent synaptic plasticity would be interesting to explore in further research.

Might it be that the processing of information about novel environments is reserved for the tri-synaptic pathway? Or is it the case that different molecular and electrophysiological mechanisms account for the processing of the same spatial information? These are further questions to be addressed in a more detailed way, possibly engaging multiple recording sites, to reveal a better understanding of hippocampal network activity as a response to general or detailed changes in the spatial environment.
5 Reference List


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6. Appendix

6.1 Curriculum Vitae

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Born in Razgrad, Bulgaria, on April 06, 1979
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Languages: Turkish, Bulgarian, English.

Educational and Research Background:

Sept. 2006 – 2010:

PhD studies at the Department of Experimental Neurophysiology, Medical Faculty;
registered student within the International Graduate School of Neuroscience, Ruhr University Bochum;
fellow within the Marie Curie Early Stage Training Programme, NovoBrain.
Appendix

**Sept. 2008:** Participated “Bordeaux PENS Training Center European Synapse Summer School”

**2006:** MSc, thesis: “The effects of Medial Septum / VDB lesions on Behavioural Despair and Learning and Memory “, Bogazici University Institute of Biomedical Engineering, Istanbul, Turkey

**2002-2006:** Research Assistant at Bogazici University, Psychology Department, Psychobiology Laboratory, Istanbul, Turkey

**1998-2002:** BSc Molecular Biology and Genetics, Bogazici University, Istanbul, Turkey

**Research and Methodological Skills:**

- Stereotaxic implantation of electrodes and cannula in rat brain
- In vivo electrophysiology of rat hippocampus
- Electrolytic, laser and chemical stereotaxic lesions of rodent brain
- Intracerebral injections and neuropharmacology of rodent brain
- Histochemical and immunocytochemical staining
- Behavioral paradigms (learning, despair, anxiety)
- Animal handling
- Western blotting procedures and analysis
Appendix

Peer Reviewed Publications:


Recent Poster Abstracts:


6.2 Acknowledgements

I would like to thank my supervisor Prof. Dr. Denise Manahan-Vaughan for her excellent supervision at every step of my thesis. Without her enormous expertise, understanding, and endless patience I would not be able to conduct this thesis.

I would like to express my special thanks to PD Dr. Suchan for his kind agreement to advise my work.

This work would not be possible without a financial support from NovoBrain Programme (Marie Curie Mobility Action Early Stage Training). I would like to express my warmest thanks to Dr. Sabine Dannenberg for her valuable efforts in coordination of the programme and immediate help on executive issues.

My warmest thanks for Jens Klausnitzer, Dr. Anne Kemp and Dr. Hardy Hagena for the valuable help they provide me about experimental and technical issues. Thanks to Beate Krenzek who was so good to conduct the histology.

I am especially thankful to Mrs. Ursula Heiler, Mrs. Heide Brusis and Mrs. Gisela Stephan who always provided vital help in administrative issues.

And my colleagues and friends Marion Andre, Arne Buschler, Jeremy Goh, Jana Kenney, Valentina Kopp, Tanja Novkovic, Honghong Yang, Sije Zhang and everybody in the lab; thanks a lot for your great support! Working with you was a great pleasure, let’s do it again in the future!

My lovely mom and dad, I would not be able to start or complete this study without your enormous efforts, thank you very much! This thesis is dedicated to you.

Burteçin’im, I feel so lucky to have you and your extensive knowledge in my side. Thank you and our lovely son Güneş for being in my life! All my efforts are dedicated to YOU.