Interactions between the entorhinal cortex and hippocampal formation
Kloosterman, F.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 4

Two re-entrant pathways in the hippocampal-entorhinal system

Fabian Kloosterman, Theo van Haeften and Fernando H. Lopes da Silva

submitted to: Hippocampus
ABSTRACT

The entorhinal cortex has long been recognized as an important interface between the hippocampal formation and the neocortex. The notion of bi-directional connections between the entorhinal cortex and the hippocampal formation have led to the suggestion that hippocampal output originating in CA1 and subiculum may re-enter hippocampal sub-fields via the entorhinal cortex. To investigate this, we used simultaneous multi-site field potential recordings and current source density analysis in the entorhinal cortex and hippocampal formation of the rat in vivo. Under ketamine/xylazine anesthesia we found that repetitive stimulation of subiculum or Schaffer collaterals facilitated entorhinal responses, such that a population spike appeared in layer III. In addition, a current sink in stratum lacunosum-moleculare of area CA1 was found, that followed responses in the entorhinal cortex, indicating re-entrance into this area. Responses indicating re-entrance in the dentate gyrus were not found under ketamine/xylazine anesthesia, but were readily evoked under urethane anesthesia. Re-entrance into CA1 was also encountered under urethane anesthesia. These results suggest that parallel, but possibly functionally distinct connections are present between the output of the hippocampal formation and cells in layer III and II of the entorhinal cortex that project to area CA1 and the dentate gyrus, respectively.
Within the temporal lobe memory system, the entorhinal cortex constitutes a major interface between the hippocampal formation and the neocortex. Highly integrated multimodal cortical information reaches the superficial layers of the entorhinal cortex via surrounding cortices (Burwell and Amaral, 1998) and these layers, in turn, distribute the information to all sub-fields of the hippocampal formation (Dolorfo and Amaral, 1998a, Steward, 1976, Steward and Scoville, 1976, Tamamaki, 1997. Witter, 1993). Hippocampal output to the entorhinal cortex arises from area CA1 and subiculum and terminates predominantly in deep layers (chapter 2, Köhler, 1985). Neurons in deep layers project to neocortical areas (Insausti et al., 1997), but they also distribute axon collaterals into the superficial layers (Dolorfo and Amaral, 1998b, Köhler, 1986, 1988, van Haeften et al., 2003). In a recent study we concluded that this deep-to-superficial layer connection likely consists of a primarily excitatory projection onto both principal neurons and inhibitory interneurons present in layers I-III (van Haeften et al., 2003). Previously, we found that stimulation of hippocampal output structures activates entorhinal deep layer neurons, which in turn transmit the activity to superficial entorhinal layers (chapter 3). This communication between deep and superficial layers may form an essential link in a circuit that can mediate re-entrance of activity into the hippocampal formation. Re-entrant circuits supporting reverberatory activity provide one possible mechanism contributing to persistent neuronal activity, which is supposed to be one of the prerequisites for working memory.

Deadwyler et al. (1975) were the first to show, employing in vivo field potential recordings, that Schaffer collateral stimulation could evoke a long latency synaptic response in the dentate gyrus, that was dependent on a functional entorhinal input to the hippocampal formation. This was confirmed by Wu et al. (1998), and these data suggest that layer II neurons that project to the dentate gyrus can be activated by hippocampal output. Yet, it is not known whether, and in what way, deep entorhinal layers participate in this process of re-entrance. Very recently, while preparing this manuscript, another study was published in which re-entrance into area CA1 was investigated in the guinea pig (Bartesaghi and Gessi, 2003). However, in this study the dorsal hippocampal commissure, mainly containing fibers that originate in the presubiculum and target cells in the superficial layers of the entorhinal cortex, was stimulated.

Here we focused on the activity in the entorhinal cortex that mediates re-entrance of activity into the hippocampal formation and particularly on the deep-to-superficial layers communication that is required for the phenomenon of re-entrance. To achieve this goal we investigated the main hippocampal output by stimulating directly the subiculum and/or the Schaffer collaterals. Moreover, we asked whether in addition to the projection from entorhinal layer II to the dentate gyrus (DG), the parallel projection from entorhinal layer III to the hippocampal formation (CA1, Subiculum) could also be engaged in transmitting hippocampal
output back to the hippocampal formation, and we established whether the type of anesthesia has influence on the occurrence of re-entrance in these two pathways.

Main differences and similarities between these previous studies and ours are discussed.

**MATERIALS AND METHODS**

**Surgery**

Female Wistar rats (180-250 gram) were anesthetized with a mixture of ketamine and xylazine (1.0-1.5 ml intra-peritoneally, 4:3 mixture of 10% solution of Ketaset Aresco, Boxtel, The Netherlands, and 2% solution of Rompun, Bayer, Brussels, Belgium) or with urethane (1.5 g /kg bodyweight) intra-peritoneally. Surgical procedures and electrode placement, optimized in order to get reliable evoked field potentials, were similar as described previously (chapter 3). Briefly, a 16-channel silicon probe (100 μm inter-electrode spacing; kindly provided by the University of Michigan Center for Neural Communication Technology sponsored by NIH NCRR grant P41-RR09754) was positioned in the dorsal part of the medial entorhinal cortex; such, that it penetrated all lamina approximately perpendicularly. A second 16-channel silicon probe was positioned into the hippocampal formation, such that it covered both area CA1 and the superior blade of the dentate gyrus. Bipolar stimulation electrodes were positioned in the dorsal subiculum and in the Schaffer collaterals in area CA3.

![Figure 4.1](image)

**Figure 4.1** Typical evoked responses in the entorhinal cortex after hippocampal output stimulation. A: Scheme of the major connections in the hippocampal-entorhinal circuitry that are involved in the generation of evoked responses in the entorhinal cortex after stimulation of subiculum or Schaffer collaterals. B,C: Example traces of field potential responses (left) and corresponding current source densities (CSD, right) in entorhinal layers V, III and II after stimulation of subiculum (SUB) or Schaffer collaterals (SCHAF). Recordings were made with a 16-channel silicon probe in all layers of the entorhinal cortex. Typical responses are labeled and the numbers between brackets correspond to the pathways labeled in A, which are involved in the generation of these responses. For further explanation, see Results. In this figure and all other figures an asterisk indicates the time of a stimulus. Abbreviations; ps, layer V population spike; w3, layer III wave; a, antidromic spike. For other abbreviations, see list.
Field potential recording

Wideband signals were amplified 200x by custom amplifiers and digitized through a CED 1401 with 32-channel simultaneous sample-and-hold extension board (Cambridge Electronic Design, Cambridge, UK). In a second set of experiments, digitization of amplified signals was performed using a 64-channel National Instruments computer board. Brief trains of isolated, constant current stimuli were applied to subiculum or Schaffer collaterals at 5-40 Hz.

Histology

At the end of an experiment, the locations of the stimulation and recording sites were marked by an electrolytic lesion (stainless steel electrodes: two 400 ms positive current pulses of 400 μA; silicon probe: injection of two 15-20 μA positive current pulses into the two outer channels for 10 seconds). Subsequently, the rat was transcardially perfused with saline and fixation solution (4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4). The brain was removed and immersed in fixation solution for at least 24 hours. Next, the brain was immersed in 20% glycerol, 2% dimethyl sulfoxide (DMSO) in 100 mM phosphate buffer, pH 7.4, for cryoprotection. Sagittal sections (40 μm thick) were cut on a freezing microtome, immersed in gelatin solution and mounted on slides, and finally stained with cresyl violet and inspected under a microscope.

Data analysis

Current source density analysis was performed by estimating the 2nd order spatial derivative of the laminar field potential profiles by a differencing procedure:

\[
CSD(h,t) = \frac{\sigma_h(\Phi(h-n\Delta h,t) - 2\Phi(h,t) + \Phi(h+n\Delta h,t))}{(n\Delta h)^2}
\]

CSD(h,t) is the current source density at fixed time t and depth h, \(\Phi(h,t)\) is the average field potential at time t and depth h, \(\Delta h\) is depth interval (100 μm), \(\sigma_h\) is tissue conductivity (assumed constant). The parameter n defines the amount of spatial smoothing applied to the data (Ahrens and Freeman, 2001; Freeman and Nicholson, 1975). Here, we used spatial smoothing (n=2) for CSD analysis of entorhinal recordings and no spatial smoothing (n=1) for CSD analysis of hippocampal recordings. CSD is presented in arbitrary units (mV/mm²).

To give a crude estimate of the CSD at the two extreme recording sites in layer I of EC, we introduced two fictive recording sites superficial to the most extreme recording site and assumed that the field potential does not change between the fictive sites and actual extreme recording site (Ahrens and Freeman, 2001).
Chapter 4

Figure 4.2 Subiculum evoked PS-W complex in the entorhinal cortex is facilitated by repetitive stimulation. A. Field potential responses in entorhinal layers I, II, III and V after several stimuli (P1, P2, P4 etc.) of a 10 Hz stimulus train applied to subiculum. Both the layer V population spike (indicated by ‘ps’) and the layer III wave (indicated by ‘w3’) were enhanced during a 10 Hz stimulus train in subiculum. During the train a biphasic wave appeared in layer I (indicated by ‘wl’), which also facilitated. The antidromic spike in layer II (indicated by ‘a’) did not show significant facilitation or depression. B. Detailed laminar profiles of the PS-W complex evoked by the 1’s and 10’s stimulus of a 10 Hz train (same experiment as in A). Notice the sharper trough of the layer III wave and the appearance of a positive-negative wave in layer I after the 10’s stimulus. C. Contour plots of the current source densities associated with the field potential responses in B. Difference between contour lines is 10 mV/mm², zero-contour is not shown. Sources are shown as dashed lines. D. Example of the development of the layer V population spike (circles) and layer III wave (triangles) amplitudes during stimulus trains applied to subiculum at 20 Hz, 10 Hz or 5 Hz.
RESULTS

Under ketamine/xylazine anesthesia, a single stimulus applied to the subiculum (SUB) or to the Schaffer collaterals (SCHAF) evoked characteristic field potentials and current source densities in the entorhinal cortex (fig. 4.1, see also chapter 3). In the entorhinal cortex, the main components of the evoked responses consisted of a layer V population spike, and a negative wave in layer III (collectively termed *PS-W complex*). SUB stimulation evoked an *early PS-W complex* due to the direct activation of the subicular-entorhinal pathway (fig 4.1A,B). SUB stimulation could also elicit a long latency *PS-W complex*, due to stimulation of the perforant path that courses through subiculum, which leads to activation of the hippocampal tri-synaptic pathway, eventually generating a *delayed PS-W complex* in the entorhinal cortex (for an example of a delayed *PS-W complex* see fig. 4.5). Alternatively, indirect activation of hippocampal output from CA1/SUB to the entorhinal cortex by SCHAF stimulation could also evoke a *PS-W complex* (fig 4.1A,C), with a latency intermediate to the early and delayed *PS-W complex* evoked by SUB stimulation.

Previously, we showed that the layer III responses during the *PS-W complex* were mediated by deep-to-superficial layer connections (chapter 3). Since neurons in entorhinal layer III give rise to a major projection to CA1 and subiculum (Steward and Scoville, 1976), we asked whether it would be possible for hippocampal output to re-enter these sub-fields. We assumed that in the case of re-entrance, SUB or SCHAF evoked responses in hippocampal sub-fields should appear later than the *PS-W complex* in the entorhinal cortex, with a latency difference that is compatible with the delay introduced by an additional pathway. However, we never found such a response after a single stimulus applied to SUB or SCHAF. Therefore, we used repetitive stimulation to probe the occurrence of re-entrance in the hippocampal-entorhinal system.

Repetitive stimulation of hippocampal output enhances entorhinal responses

To analyze the effects of repetitive stimulation, we chose frequencies of 5-40 Hz, which overlap with the range of paired pulse intervals investigated in our previous study (chapter 3). During repetitive stimulation at 10 Hz in SUB the amplitudes of the layer V population spike and the layer III wave in the entorhinal cortex were enhanced (fig 4.2A,B, indicated by ps and w3 respectively). In addition, in layer I a biphasic positive-negative wave appeared after the first few stimuli and was facilitated after subsequent stimuli (fig 4.2A,B, indicated by w1). The peak of the negative component of this layer I component was 5-11 ms after the peak of the layer III wave. The layer III wave did not only increase in amplitude, but its trough became sharper, such that we may describe this as a layer III population spike (fig. 4.2A,B). CSD analysis showed that this corresponded to a sharp transient sink superimposed onto the slower sink of the layer III wave (fig. 4.2C). This layer III population spike appeared to propagate towards layer II. It appeared that at the time of the facilitated layer III wave, a
Figure 4.3 Summary of the dynamics of the layer V population spike (A), layer III wave (B) and long latency stratum lacunosum-moleculare response in CA1 (C) during a stimulus train applied to subiculum at 20 Hz (top), 10 Hz (middle) and 5 Hz (bottom). Dots represent individual measurements from several experiments; the line connects the averages at each stimulus. Amplitudes were normalized to the maximal value reached during the train. Note the delayed enhancement of the CA1 SLM responses compared with that of the other two events.

large negative-going potential was also present in deep layers, following the population spike, which was not present after a single stimulus (fig. 4.2B, also indicated by an arrow in fig. 4.2A for P10). However, CSD analysis revealed that in deep layers a current sink was only generated during the layer V population spike, but not during the later large negativity (fig 4.2C), demonstrating that this latter potential was due to volume conduction of layer III responses.

Facilitation of the layer III wave was stronger than that of the layer V population spike (fig. 4.2A, D). The dynamics of the facilitation were different for deep and superficial layer responses and also varied for different stimulation frequencies (fig 4.2D). To pool the data from several experiments, the amplitudes were normalized to the maximal amplitude within a train applied to SUB (fig 4.3A,B). At a stimulation frequency of 10 Hz, the layer V population spike reached a plateau level already after the second stimulus, whereas the amplitude of the layer III response continued to increases until the 3rd-5th stimulus (fig 4.3B and fig 4.2D). Stimulation at 20 Hz resulted in robust facilitation during the first few stimuli for both the layer V spike and layer III wave. After the initial few stimuli of the train the layer V population spike tended to de-facilitate slightly, whereas the amplitude of the layer III wave remained at the same level (fig 4.3A,B). A very similar pattern was observed at 40 Hz.
stimulation (not shown). With 5 Hz stimulation responses were facilitated, albeit at a slower rate (fig. 4.3A,B and 4.2D). That is, the layer V population spike reached a plateau level only late during the train, and the layer III wave did not reach a plateau level at all during the ten stimuli applied.

Delayed PS-W complexes evoked by SUB stimulation and SCHAF stimulation evoked PS-W complexes were also subject to facilitation. In all these cases the layer III wave became sharper and current source density analysis demonstrated the presence of a layer III population spike. In addition, a layer I positive-negative wave was frequently present, similar as described above.

**Repetitive stimulation of hippocampal output evokes a long latency sink in stratum lacunosum-moleculare of area CA1**

Field potential responses in CA1 and dentate gyrus evoked by a single stimulus in either SUB or SCHAF reflected the activation of both perforant path fibers and intra-hippocampal pathways (Kloosterman et al., 2001, Leung et al., 1995, Wu et al., 1998). During repetitive SUB or SCHAF stimulation, a long latency potential appeared in the hippocampal formation (figs 4.4A and 4.5). This response had a fixed time relationship to the PS-W complex in the entorhinal cortex, such that it started 4-11 ms after the peak of the layer III wave of either the SUB evoked early PS-W complex (n=5/8, fig 4.4A), the SUB evoked delayed PS-W complex (n=5/5, fig 4.5) or the SCHAF evoked PS-W complex (n=4/5, fig. 4.4B). The long latency hippocampal response appeared as a negative wave in the dentate gyrus (including the hilus) and close to the fissure in area CA1, and reversed to a small positive wave in stratum radiatum (RAD) and the pyramidal layer (fig. 4.4C1,C2). CSD analysis showed that the long latency response was associated with a current sink close to the fissure, most likely in the stratum lacunosum-moleculare (SLM) of CA1, and a source in RAD (fig 4.4C3). The laminar field potential profile of this response did not differ between SUB and SCHAF stimulation. However, as illustrated in figure 4.4C2, it clearly differed from the profile of the dentate gyrus fEPSP after SUB stimulation (due to activation of passing fibers of the perforant path). In addition, it also differed from the profile of the fEPSP in CA1 evoked by low intensity stimulation of the Schaffer collaterals (fig 4.4D1). These differences can also be clearly seen in the CSD profiles (fig 4.4C3,D2). The fact that the source that accompanied the long latency sink was located in RAD, supports the notion that the response involved the distal dendritic region of CA1 neurons, rather than the outer molecular layer of the dentate gyrus. In a few cases lesions were made at the outer recording sites of the hippocampal probe (see Methods), and the histology in these cases reinforced the interpretation that the sink was located in SLM (fig 4.5B).

The SLM sink found after the early PS-W complex evoked by SUB stimulation was facilitated during a stimulus train. We compared the dynamics of the long latency SLM sink (fig. 4.3C) to the layer V and III components of the early PS-W complex after SUB
stimulation (fig. 4.3A,B). The dynamics of the SLM sink appeared to resemble the dynamics of the layer III wave more than the dynamics of the layer V population spike (particularly evident for stimulus trains at 20 and 10 Hz).
Re-entrance in the hippocampal-entorhinal system

Re-entrance into area CA1 and dentate gyrus under urethane anesthesia

As mentioned above, under ketamine/xylazine anesthesia there was no indication of a long latency event in the dentate gyrus that could point at re-entrance of activity into this structure. Previously, however, long latency potentials in the dentate gyrus have been reported after SHCAF stimulation under urethane anesthesia (Deadwyler et al., 1975, Wu et al., 1998). We asked whether there was a difference between urethane and ketamine/xylazine anesthesia for inducing re-entrance of neuronal activity into the hippocampal formation.

SUB stimulation resulted in a long-latency negative-going wave in the hippocampal formation following a PS-W complex in the entorhinal cortex, similar as under ketamine/xylazine anesthesia (n=5/6, fig 4.6A). CSD analysis showed that an associated current sink was located in stratum lacunosum-moleculare of CA1 and a current source was located in stratum radiatum (fig 4.6B). In addition, a long-latency small positive-going potential was found in the hilar region, which reversed in the molecular layer (n=4/6, fig 4.6A,B). It is important to note that this response was not observed under ketamine/xylazine anesthesia (e.g. compare figures 4.4A and 4.6A, stimuli P10). CSD analysis demonstrated that the long-latency DG potential was associated with a current sink in the molecular layer of the dentate gyrus and a current source in the granule cell layer (fig 4.6B). This sink-source configuration was identical to that of perforant path evoked responses in the dentate gyrus (fig. 4.6B). A long-latency dentate gyrus positivity and associated current sink in the molecular layer could also be found after SCHAF stimulation (n=7/7). In only one case, SCHAF stimulation evoked a small long-latency sink in SLM of CA1.

Since we observed long latency potentials, indicative of re-entrance, in the dentate gyrus of urethane, but not ketamine/xylazine anesthetized animals, we asked whether differences could be observed in the evoked responses in the entorhinal cortex. In most cases this was,
Figure 4.5 A. A long latency hippocampal response in stratum lacunosum-moleculare can follow a delayed PS-W complex (dPS-W, ps: layer V population spike, w3+ws: layer III population spike superimposed onto layer III wave) evoked by activation of the perforant path in subiculum. An overlay of field potential responses recorded using a 16-channel probe in the hippocampal formation shows that subiculum stimulation evoked an antidromic spike in CA1 (a), a large positivity in the hilus (b) with superimposed population spike (c), and tri-synaptic EPSP in stratum radiatum of area CA1 (d). The inset shows the long latency SLM response (e) at larger scale. B. Sagittal Nissl-stained section showing lesions (arrowheads) of the outer recording sites of a 16-channel probe in the hippocampal formation. The complete recording track is reconstructed by linear interpolation between the two lesions. In panel C, the depth profile of the long latency SLM response and the associated current source density profile are shown. Notice that the current sink was located in SLM, and the source in stratum radiatum.

however, not the case. Under urethane anesthesia, a single stimulus in subiculum evoked a PS-W complex in the entorhinal cortex (fig. 4.6A), that was similar to those evoked under ketamine/xylazine anesthesia (fig. 4.2A). Also, under urethane the PS-W complex facilitated during a brief train of stimuli, in some cases leading to a layer III population spike (figs 4.6A, 4.7A,B). In a few cases after Schaffer collateral stimulation it appeared that the current sink in layer III extended further into layer II than was ever observed under ketamine/xylazine anesthesia (fig. 4.7D, gray arrow), which may indicate additional involvement of layer II cells.

DISCUSSION

In the present study, we have demonstrated in the rat in vivo, that entorhinal field potential responses evoked by stimulation of subiculum were enhanced during a stimulus train, which was reflected in the emergence of a layer III population spike. Furthermore, we obtained evidence for re-entrance of activity into the hippocampal formation via the entorhinal cortex upon subiculum or Schaffer collateral stimulation: a late current sink in
Re-entrance in the hippocampal-entorhinal system

Figure 4.6. A. Under urethane anesthesia, repetitive 10 Hz subiculum stimulation evoked long latency responses in both SLM (closed arrow) and dentate gyrus (open arrow), both following the PS-W complex in the entorhinal cortex. B. Full laminar profile of long latency hippocampal response after the 10th stimulus shown in A. Field potential depth profiles (B2) and associated current source densities (B3) are shown for the time points indicated by the vertical dashed lines. These time points indicate peaks of the early perforant path evoked response in the dentate gyrus and the long latency responses in the dentate gyrus (21.5 ms) and CA1 SLM (31.0 ms). In C, Field potential depth profile and associated current source densities of the Schaffer collateral evoked EPSP in CA1 is shown for the same experiment for comparison.
Figure 4.7. Evoked responses in the entorhinal cortex under urethane anesthesia. A full laminar profile of subiculum evoked field potentials following the 10th stimulus in a 10 Hz train (same experiment as fig. 6) is shown in A. In B and C, contour plots of current source densities associated with the field potentials following the 10th (B) and 20th (C) stimulus are plotted. Difference between contour lines is 6.7 mV/mm²; zero-contours are not plotted. Sources are indicated by dashed lines. D. Contour plot of the current source density associated with SCHAF evoked responses following the 10th stimulus in a 10 Hz train (same experiment as A-C). The gray arrow indicates the extension of a current sink into layer II.

A stratum lacunosum-moleculare of area CA1 appeared, that followed entorhinal responses, and - a late current sink appeared in the molecular layer of the dentate gyrus following entorhinal responses, but this sink only occurred under urethane anesthesia and not under ketamine/xylazine anesthesia.

**Frequency facilitation of entorhinal responses**

During a paired pulse protocol, the subiculum evoked entorhinal responses showed facilitation, with maximal effect at intervals ranging from 25-100 ms, but no depression (chapter 3). Our present data show that a stimulus train applied to subiculum resulted in entorhinal responses being even further enhanced, particularly in layer III, where a population spike emerged. This enhancement was stronger at 10-20 Hz than at 5 Hz, which is in line with the facilitation observed in a paired pulse protocol (chapter 3). The dynamics were different for layer V and layer III responses and also for different stimulus train frequencies. This suggests that the facilitation of the layer III response is not completely determined by the facilitation of the layer V population spike. We did not investigate the mechanism of the facilitation, but we may suggest that the observed facilitation in EC layer V after subiculum stimulation may be due to a decrease in feed-forward inhibition jointly with an increase in glutamate release, both involving presynaptic metabotropic glutamate receptors (Evans et al., 1994).
Re-entrance in the hippocampal-entorhinal system

2000; Woodhall et al., 2001). Since the deep-to-superficial layer projection predominantly consists of excitatory contacts onto principal neurons and inhibitory interneurons in layer I-III (van Haeften et al., 2003), similar mechanisms may account for the additional facilitation in superficial layers that is not explained by the facilitation of deep layer responses.

Signs of re-entrance in SLM of area CA1

Following the subiculum evoked responses in the entorhinal cortex, a response was observed in stratum lacunosum-moleculare (SLM) of area CA1. The laminar field potential profile and associated current source density profile are similar to what could be expected based on computer simulations (Leung, 1995), and to responses evoked by direct entorhinal cortex stimulation (Leung et al., 1995). Furthermore, current density profiles of the SLM response were clearly different from perforant path evoked responses in the dentate gyrus or Schaffer collateral evoked responses in stratum radiatum (RAD) of area CA1. Lastly, histology indicated that this response is likely generated in SLM.

How are the CA1 SLM responses generated? Our results suggest that entorhinal layer II to CA1 projections mediated the SLM response, based on the following observations: 1. a population spike emerged in layer III during train stimulation, 2. the SLM sink followed the layer III population spike with a latency consistent with the delay introduced by a single synapse and 3. entorhinal layer III and SLM responses during train stimulation have similar dynamics. These findings taken together show that a functional re-entrance pathway exists from the output structures of the hippocampal formation, via the entorhinal deep layers, to entorhinal layer III principal neurons that project to area CA1.

Entorhinal layer III neurons mainly form excitatory contacts with distal dendrites of pyramidal neurons and interneurons in SLM (Desmond et al., 1994, Witter et al., 1992). The current sink that was observed in SLM likely represents excitation of the distal dendrites of CA1 pyramidal neurons, which, however, was not sufficient to discharge the pyramidal neurons. This corroborates other studies that showed a predominant feed-forward inhibition exerted by the direct entorhinal input and only a small direct excitation that is not capable of depolarizing the cell soma above threshold for action potential generation (Empson and Heinemann, 1995a, b, Leung et al., 1995, Leung, 1995, Levy et al., 1995, Paré and Llinas, 1995, Soltesz, 1995). Some studies, however, did show discharge of CA1 pyramidal neurons upon stimulation of the direct entorhinal input (Doller and Weight, 1982, Yeckel and Berger, 1995) and similar results have been obtained for the entorhinal cortex input to subiculum (Naber et al., 1999). In addition, entorhinal input to SLM may induce pyramidal cell firing when inhibition is blocked (Empson and Heinemann, 1995b).

In a recent paper, Bartesaghi and Gessi (2003) reported that stimulation of the dorsal hippocampal commissure in the guinea pig resulted in a late current sink in SLM of area CA1, which may be mediated by re-entrant activity in the hippocampal-entorhinal circuitry. Our
present data is in agreement with their conclusions and shows that re-entrance into area CA1 can also be found in rat. There are, however, a number of differences between the Bartesaghi and Gessi study and ours, which we should address. Stimulation of the dorsal hippocampal commissure is a different way of activating hippocampal output, since the commissural presubicular input to superficial layers of the entorhinal cortex is initially activated, followed by sequential activation of the dentate gyrus, CA3, CA1 and subiculum, which finally results in responses in deep and superficial layers of the entorhinal cortex (Bartesaghi et al., 1988, 1989). In this protocol, the superficial layers are activated twice and it could be argued that the initial response evoked by the commissural presubicular input influences the later entorhinal responses. In contrast, we stimulated directly the entorhinal deep layers since the stimulus was applied to SUB or Schaffer collaterals. It could be argued for SUB stimulation that superficial entorhinal circuits might have been stimulated antidromically, but this drawback does not hold for SCHAF stimulation. Another difference relates to the frequencies used for stimulation. Bartesaghi and Gessi used frequencies ranging from 1 to 5 Hz, and found that 3 Hz was optimal. In contrast, we used frequencies ranging from 5 to 20 Hz, and found that the re-entrance into CA1 was stronger at 10 and 20 Hz than at 5 Hz. Whether these contrasting findings are due to the use of different anesthetic agents, i.e. sodium thiopental in the Bartesaghi and Gessi study and ketamine/xylazine in our study, or to the distinct stimulation protocols or reflect a difference between rat and guinea pig, is unclear.

Signs of re-entrance in the dentate gyrus

Contrary to the sink in CA1 SLM, a long latency response in the dentate gyrus indicative of re-entrance of activity into this structure was not found under ketamine/xylazine anesthesia. The latter was, however, found under urethane anesthesia. Bartesaghi and Gessi (2003) reported that re-entrance into the dentate gyrus was not present in the guinea pig anesthetized with sodium thiopental. Apparently, layer II stellate neurons projecting to the dentate gyrus do not fire upon hippocampal output stimulation under these conditions. One possible explanation would be that layer II neurons do not receive excitatory input from entorhinal deep layers. This is not likely, however, since anatomical studies have indicated that an excitatory projection to layer II cells is present (van Haeften et al., 2003). Another explanation could be that layer II stellate neurons projecting to the dentate gyrus are under strong feed-forward inhibitory control. Nevertheless, under urethane anesthesia re-entrance into the dentate gyrus is readily evoked (present study; Deadwyler et al., 1975, Wu et al., 1998), and it was shown to depend on a functional input from the entorhinal cortex.

What possible mechanism accounts for the differential sensitivity of re-entrance into the dentate gyrus to various anesthetic agents? It is likely that the mechanism lies at the level of entorhinal layer II neurons, rather than at the level of the dentate gyrus. An explanation could lie in the (anti)agonist effects of these anesthetic drugs on different neurotransmitter systems. Ketamine is a competitive blocker of NMDA receptors and xylazine is an agonist of \( \alpha_2 \)
adrenergic receptors. Sodium thiopental, on the other hand, is a barbiturate that enhances GABAergic synaptic transmission. Little is known about the anesthetic mechanism of urethane, but a recent study demonstrated that at anesthetic doses it might have a modest effect on a variety of ligand-gated ion channels, including AMPA, NMDA and GABA-A receptors (Hara and Harris, 2002). Further study is needed to elucidate the mechanisms underlying these novel observations. This would also shed light on the physiological differences between the two re-entrance pathways via layer II to DG and via layer III to CA1 SLM, respectively.

**Functional relevance of re-entrance**

What could be the functional consequences of re-entrance pathways in the hippocampal-entorhinal circuitry that connect hippocampal output (entorhinal layer V) with hippocampal input (entorhinal layers II/III)? From the point of view of the hippocampal formation the outputs from CA1/subiculum can activate, via entorhinal deep layers, cells in superficial entorhinal layers II and III which project to the dentate gyrus and area CA1/subiculum respectively, as we have demonstrated here. Thus, in entorhinal superficial layers, integration of hippocampal output and cortical input may occur and serve a comparator function (Lorincz and Buzsaki, 2000, Naber et al., 2000). Or, in terms of the computational model put forward by Lorincz and coworkers (Lorincz, Buzsaki, 2000; Lorincz et al., 2002), this circuit may compute the 'error' between the actual neocortical input to the entorhinal cortex and the representation stored in the hippocampal formation. Whether this comparison takes place in the connections between entorhinal deep layers and layer III (Naber et al., 2000), layer II (Lorincz and Buzsaki, 2000) or both is not clear.

Taking this a step further, we can see that the parallel projections from layer III to area CA1/subiculum and layer II to the dentate gyrus close this circuit. This could constitute the anatomical and physiological substrate for reverberation to occur, in the sense of persistent activity in these loops in the absence of external input (Wang, 2001). However, under the present experimental conditions, i.e. under anesthesia, we did not find evidence for reverberation according to this definition. Nevertheless, it has been suggested that in the subcortically denervated hippocampus of the freely moving rat, removal of tonic inhibitory influences allows reverberation of information in the entorhinal-hippocampal-entorhinal cortex circuitry (Buzsaki et al., 1989). It is still an open question whether also under physiological conditions reverberation can take place.

In conclusion, a novel aspect of this study is the demonstration that re-entrance to the hippocampal formation can occur along two parallel pathways, one to the dentate gyrus and the other one to CA1. This is in contrast to previous studies, which showed that re-entrance occurs in either one or the other pathway, but not in both simultaneously. Both re-entrance pathways are operational under urethane anesthesia, but the re-entrance pathway to the dentate gyrus was not active under ketamine/xylazine anesthesia. These distinct
pharmacological profiles suggest that these parallel pathways involve, at least partly, different neurotransmitter systems. Whether in freely moving animals, these two parallel pathways act in unison or differentially depending on behavioral conditions, is an interesting issue that merits further study.