Chapter 3

Electrophysiological characterization of inter-laminar entorhinal connections: an essential link for re-entrance in the hippocampal-entorhinal system

Fabian Kloosterman, Theo van Haeften, Menno P. Witter and F.H. Lopes da Silva

submitted to: European Journal of Neuroscience
Chapter 3

ABSTRACT

The hippocampal formation communicates with the neocortex mainly through the adjacent entorhinal cortex. Neurons projecting to the hippocampal formation are found in the superficial layers of the entorhinal cortex and are largely segregated from the neurons receiving hippocampal output, which are located in deep entorhinal layers. We studied the communication between deep and superficial entorhinal layers in the anesthetized rat using field potential recordings, current source density analysis and single unit measurements. We found that subiculum stimulation was able to excite entorhinal neurons in deep layers. This response was followed by current sinks in superficial layers. Both responses were subject to frequency dependent facilitation, but not depression. Selective blockade of deep layer responses also abolished subsequent superficial layer responses. This clearly demonstrates a functional deep-to-superficial layer communication in the entorhinal cortex, which can be triggered by hippocampal output. This pathway may provide a means by which processed hippocampal output is integrated or compared with new incoming information in superficial entorhinal layers, and it constitutes an important link in the process of re-entrance of activity in the hippocampal-entorhinal network which may be important for consolidation of memories or retaining information for short periods.
INTRODUCTION

The hippocampal formation and the adjacent parahippocampal region are important brain structures that mediate processing and storage of highly integrated sensory information. The connections between the hippocampal formation and the parahippocampal region are characterized by reciprocal pathways and multiple loops (Amaral and Witter, 1995, Lopes da Silva et al., 1990, Witter, 1993). The entorhinal cortex, one of the major constituents of the parahippocampal region, is both the main source of cortical information entering the hippocampal formation as well as one of the main destinations of processed output leaving the hippocampal formation.

According to the classic view of entorhinal-hippocampal connections, the cells giving rise to hippocampal input pathways and cells targeted by the hippocampal output pathways are segregated in different layers of the entorhinal cortex. More specifically, neurons in the superficial layers of the entorhinal cortex project to all subfields of the hippocampal formation (Canning et al., 2000, Dolorfo and Amaral, 1998a, Leung et al., 1995, Steward, 1976, Steward and Scoville, 1976, Tamamaki, 1997, Witter, 1993). In turn, neurons in the entorhinal deep layers are the main recipients of hippocampal output, that originates in area CA1 and subiculum (chapter 2, Beckstead, 1978, Köhler, 1985, Naber and Witter, 1998, Swanson and Cowan, 1977, van Haeften et al., 1995) and are the source of output fibers that project to the neocortex (Insausti et al., 1997).

Recently, an extensive associational projection from deep to superficial layers was demonstrated within the entorhinal cortex (Dolorfo and Amaral, 1998b, Köhler, 1986, 1988, van Haeften et al., 2003). Also, a small portion of the projections of subiculum and presumably CA1 to the entorhinal cortex terminates in superficial layers (chapter 2, Köhler, 1985, van Haeften et al., 1995). In the guinea pig, indirect activation of hippocampal output by stimulation of commissural presubicular fibers in the dorsal psalterium resulted in long latency responses in all layers of the entorhinal cortex (Bartesaghi et al., 1989), and it was suggested that intra-entorhinal connections were involved in the generation of the superficial entorhinal responses. Similar suggestions were made for the generation of epileptiform activity in these brain areas (Paré et al., 1992, Scharfman, 2002). It has been also assumed that intra-entorhinal communication takes place based on in vivo experiments showing delayed activation of the dentate gyrus following hippocampal stimulation (Deadwyler et al., 1975, Wu et al., 1998). How the deep entorhinal layers participate in this process, however, has not been firmly established. Indeed, the characteristics of the entorhinal deep-to-superficial communication are not yet well known. A recent ultrastructural anatomical study has provided insight in the synaptic organization of the deep-to-superficial connections, indicating that this projection is predominantly excitatory, targeting both principal as well as inhibitory interneurons in almost equal percentages (van Haeften et al., 2003). In the light of this new anatomical information, we decided to re-examine the physiology of the hippocampal-
entorhinal and intra-entorhinal circuitry in vivo in more detail. This was done using in vivo field potential recordings, current source density analysis and single unit measurements.

MATERIALS AND METHODS

Surgery
Female Wistar rats (180-250 gram, Harlan Centraal Proefdierbedrijf, Zeist, The Netherlands) were anesthetized with a mixture of ketamine and xylazine (4:3, 1.0-1.5 ml intraperitoneally, 10% solution of Ketaset Aesco, Boxtel, The Netherlands, and 2% solution of Rompun, Bayer, Brussels, Belgium). Additional doses were administered intramuscularly, if the withdrawal reflex after hind paw pinching returned. Rats were placed in a stereotaxic apparatus and kept warm with a circulating water bath. After exposure of the skull, trephine windows were made to provide access to underlying brain structures. Part of the sagittal sinus was exposed, the midpoint of which served as the medio-lateral zero-point. A stainless steel screw, driven into the frontal bone, or the stereotaxic frame served as reference for recording.

Stimulation electrodes
A bipolar stimulation electrode (60 μm insulated stainless steel, 300-600 μm vertical tip separation) was placed in the dorsal subiculum (6.0-6.2 mm posterior to bregma, 3.2 mm lateral, 2.8-3.2 mm below cortical surface) (fig. 3.1A). The coordinates of the stimulation electrode were derived from a previous anatomical study (chapter 2) in which we described a topographical arrangement of subicular projections to the entorhinal cortex and from a stereotaxic atlas of the rat brain (Paxinos and Watson, 1998). Since our recordings were performed in the dorsal part of medial entorhinal cortex (MEA, see below), we positioned the stimulation electrode in the distal half of subiculum (i.e. close to presubiculum border), although in some experiments the stimulation electrode was located in more proximal-intermediate parts of subiculum. Subiculum was stimulated using a standard paired pulse protocol with a 100 ms inter-pulse-interval and a typical repetition rate of 0.08 Hz. In some experiments, additional bipolar stimulation electrodes were positioned in Schaffer collaterals (3.2 mm posterior to bregma, 3.2 mm lateral, 3.0 mm below cortical surface) or in the entorhinal cortex (0.3-0.5 mm anterior to the transverse sinus, 4.4-4.6 mm lateral, 2.0-2.5 mm below cortical surface).

Field potential recordings
Two approaches were used to record evoked field potentials in MEA. In order to assess the distribution of evoked field potentials along the dorso-ventral axis of MEA after dorsal subiculum stimulation, a multi-channel probe (6 parallel insulated stainless steel wires of 60 μm diameter) was lowered into MEA just anterior of the transverse sinus, 5.0 mm lateral.
Intrinsic entorhinal cortical communication

Figure 3.1 Histology of stimulation and recording locations in subiculum and entorhinal cortex. A. Nissl-stained sagittal section of the hippocampal formation illustrating the stimulation site in dorsal subiculum. Arrows point to the lesions of the bipolar stimulating electrode. Lines indicate the extent of white matter and the borders of the subiculum (dashed). B. Nissl-stained sagittal section illustrating the recording track of a 16-channel silicon probe in the dorsal part of the medial entorhinal cortex. Lesions were made at the 1st, 8th, and 16th recording sites (arrowheads). Entorhinal layers are indicated with Roman numerals. Extent of white matter and the borders of layer II are drawn as lines, the lamina dissecans is drawn as a dashed line. For abbreviations in this figure and all following figures, see list.

angled at 12°-15° in the sagittal plane. Using this approach, recordings were made from 6 sites across several layers of MEA at several dorso-ventral levels (2.0-4.0 mm below the cortical surface).

To construct a detailed laminar profile of field potentials in MEA, either a glass micropipette filled with 0.5 M NaCl and 2% pontamine sky blue solution (impedance: 10-30 MW) or 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 used. The electrodes were lowered into MEA using a hydrostatic micromanipulator (Narashige, Tokyo, Japan), at a location 1.0-2.0 mm posterior to bregma, 5.0 mm lateral, angled at 50-60° in the sagittal plane. The glass micropipette was stepped down 50 or 100 μm each time in order to record the depth profile. This approach resulted in a recording track that ran almost orthogonal to the cortical layers (see fig. 3.1B). Evoked field potentials appeared similar regardless of the recording procedure used.

In some experiments, additional field potentials were recorded in CA1 and dentate gyrus using an assembly of three stainless steel wires cut at different lengths or an additional 16-channel silicon probe (100 μm spacing).

Field potentials were amplified (6-channel electrode and glass electrode: 100-500x, Axon Cyberamp, Axon Instruments, Inc., Union City, CA; 16-channel probe: 200x, custom made amplifier), low pass filtered (6-channel probe and glass electrode: cut off at 5-10 kHz; 16-channel probe: no filtering) and sampled at 10-20 kHz using a CED 1401 acquisition interface and Signal software (Cambridge Electronic Design, Cambridge, UK). 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) or with dye ejection from the micropipette (10-20 minutes repetitive (0.5 s on, 0.5 s off) negative current injection of 20-40 μA).

**Histology**

After completion of an experiment, the rat was decapitated and the brain was removed and immersed in fixation solution (4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for at least 24 hours. In some cases the rat was first transcardially perfused with saline and fixation solution and the brain was subsequently removed and stored in fixation solution. Next, the brain was immersed in 20% glycerol, 2% dimethyl sulfoxide (DMSO) in 100 mM phosphate buffer, pH 7.4, for cryoprotection. For visualizing Fe3+ ions deposited by the electrolytic lesion induced by the stainless steel electrodes, hexacyanoferrate(II) was added to the fixation solution. 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.

**Drug application**

Theta micropipettes were pulled to a final diameter of about 3 μm and filled with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 1.5-2 mM in saline, pH=8.5) and pontamine sky blue solution (2% in 0.5 M NaCl). CNQX was iontophoretically applied (20-50 μA current) to the deep layers of MEA close to the site of recording in 3 experiments. The delay between start of the drug application and the effect on the deep entorhinal field potential responses was variable, depending on the distance between the site of drug application and the recording site. The site, at which CNQX was applied, was marked with pontamine sky blue at the end of the experiment. CNQX was obtained from Tocris Cookson Ltd., Bristol, UK.

**Single unit recordings**

Single unit activity was measured using a glass micropipette (diameter 0.5-2 μm) filled with a solution of 0.5 M NaCl and 2-2.5% Neurobiotin (Vector Laboratories, Burlingame, CA). The electrode was inserted into MEA, 4.2-5.2 mm lateral, either 0.2-0.4 mm anterior to the transverse sinus (aiming at superficial layers) or 0.6-0.8 mm anterior to the transverse sinus (aiming at deep layers). Initial depth was 1-1.5 mm below the cortical surface. The electrode was slightly angled (10-12°) so that the track was parallel to the layers of MEA.

Wideband signals were recorded using an Axoclamp 2a amplifier (Axon Instruments, Union City, CA) in bridge mode. The signals were additionally amplified and filtered with a Cyberamp (Axon Instruments, Union City, CA). Filter settings for single unit activity were: 300 Hz – 10 kHz; no filter was used for the evoked field potentials. Evoked field potentials
and evoked unit responses were digitized at 10 kHz using Signal software (Cambridge Electronic Design, Cambridge, UK).

At most three tracks separated by 300-400 μm were made in one hemisphere. Units were detected along a given track and it was tested whether they responded to subiculum stimulation with a clear change in firing rate, either an increase or a decrease. For those units for which a clear change in firing rate was found, input-output curves were made. Since decreases in firing rate are only clear for cells with a relatively high basal firing rate, this group was probably underestimated. This is especially the case for units in entorhinal deep layers, since these had generally very low background firing. We attempted to label cells that responded to subiculum stimulation using the juxtacellular labeling technique (see below). Cells were assigned to a particular entorhinal layer on the basis of labeling with neurobiotin and/or shape of the evoked field potential and/or reconstruction of the recording track.

**Juxta-cellular labeling and tissue processing**

In order to identify the exact location and morphology of recorded cells, they were labeled according to the method described by Pinault (1996). In short, after recordings were finished the micropipette was positioned as close to the cell as possible, guided by the amplitude of the recorded extra-cellular action potentials, without damaging it. Current was then injected through the micropipette (positive pulses 3-10 nA on negative baseline 1-4 nA, 0.25 sec on, 0.25 sec off), which expelled Neurobiotin from the pipette and induced the cell to fire. It most cases this protocol was applied for at least 20 minutes.

Perfusion, fixation and storage of the brain were performed as described above. Serial histological sections were cut sagittaly (40 μm thick) on a freezing microtome and collected in phosphate buffer. Locations of the stimulation sites were verified by inspecting cresyl violet stained sections containing the lesions in the microscope. Free-floating sections containing MEA were subjected to histochemistry as follows. First the sections were rinsed in phosphate buffer, pH 7.4, followed by incubation for 30 min in 0.2% peroxidase solution in phosphate buffer (pH 7.6), and rinsed again in phosphate buffer and in 0.5% Triton X-100 dissolved in TBS, pH 8.0 (TBS-TX). In order to visualize Neurobiotin, sections were incubated overnight in avidin-biotin complex (Vectastain, Vector Laboratories, Burlingame, CA) at 4°C (pH 8.0). Sections were then rinsed in TBS-TX and in Tris/HCl and subsequently reacted with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO), H2O2 and nickel ammonium sulphate. After sufficient staining was obtained, the reaction was terminated with Tris/HCl, pH 8.0. Sections taken from gelatin solution (0.2% in 50 mM Tris/HCl, pH 8.0) were mounted on glass slides and coverslipped with Entellan (Merck, Darmstadt, Germany).
Data analysis

Recorded signals were processed and analyzed off-line using Signal (Cambridge Electronic Design, Cambridge, UK) and Matlab (The MathWorks, Inc.) software. Averaged evoked field potential responses were constructed from 4-16 sweeps. DC levels and low frequency drift present in the recordings were removed by subtracting the best linear fit. One dimensional current source density (CSD) was estimated by approximation of the 2nd order derivative in space using the following formula:

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

CSD(h,t) is the current source density at fixed time t and depth h, \(F(h,t)\) is the average field potential at time t and depth h, \(Dh\) is depth interval (100 \(\mu\)m), \(Sh\) is tissue conductivity. The parameter \(n\) defines the amount of spatial smoothing applied to the data (Ahrens and Freeman, 2001, Freeman and Nicholson, 1975). In this study we used \(n=2\). CSD is presented in arbitrary units (mV/mm\(^2\)).

To give a crude estimate of the CSD at the two extreme recording sites in layer I of MEA, 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). In the figures showing CSD depth profiles, the crude CSD estimates at the two most superficial recording sites are indicated by dashed lines.

We should emphasize that one-dimensional CSD analysis assumes that the major extracellular current flows parallel to the recording track. Due to the complicated geometry of the entorhinal cortex this assumption can hardly be fulfilled in practice. Nevertheless, we found that a given subiculum stimulation site results in a rather wide domain in MEA with similar evoked responses (see Results) and that the recordings made at different angles with respect to the MEA layers displayed a strong similarity, what indicates that our CSDs were not much affected by the relative obliqueness of some tracks. This appears to indicate that the MEA domains that were activated by the subicular stimulation occupied a rather wide extension of the entorhinal cortex and that no significant contribution from transversal currents was present, as was also shown before in a similar study in the cat (van Groen et al., 1987).

Another issue concerning the application of CSD analysis is the assumption that tissue conductivity is homogeneous. For hippocampal area CA1 small gradients in conductivity have been measured (Holsheimer, 1987), but it is generally assumed that these do not significantly alter the CSD profile. For the entorhinal cortex the tissue conductivity is unknown and is usually taken as a constant (Ahrens and Freeman, 2001, van Groen et al., 1987). Considering the inhomogeneity of cell density in the entorhinal cortex (particularly layer II and the lamina dissecans), it may be inferred that gradients in conductivity would exist. Nevertheless, we assumed that these gradients are small and do not affect the CSD profiles substantially.
RESULTS

Dorso-ventral profile of subiculum evoked field potentials in MEA

To determine the extent of the area within MEA that is activated by a given stimulation in dorsal subiculum, we recorded evoked field potentials at several locations in MEA using an array of electrodes placed approximately parallel to the cortical surface. We found that the most prominent responses with similar amplitude and waveform were present in the dorsal half of MEA. Ventral to this region no or very small responses were detected. Therefore, all experiments described below aimed at recording from the dorsal part of MEA.

Subiculum evoked responses in the entorhinal cortex

Histological verification of the electrode track showed that in 15 cases (n=7 for glass micropipette; n=8 for 16-channel silicon probe) recordings were performed in all layers of the entorhinal cortex (fig. 3.1B). Subiculum stimulation induced a typical spatio-temporal pattern of field potential responses in MEA as shown in figure 3.2.

In the majority of experiments (n=12/15), the first events after subiculum stimulation were two short latency negative deflections (n1, n2; fig. 3.2). The amplitude of n1 was small in layer III, but increased abruptly to a maximum in layer II. Here, the peak latency was 2.0 ± 0.1 ms (range 1.6-2.9 ms). Following the n1 event, a second negative deflection appeared in layer V and III (n2, fig. 3.2) at a latency of 2.4 ± 0.1 ms (measured in layer III, range 2.1-2.9 ms). The amplitude of the n2 event was small throughout layer III and V.

A slower, longer latency, negative wave was observed in layer I close to the border with layer II (n3; latency of trough 8.7 ± 0.9 ms, n=11/15, fig. 3.2). This n3 event was only observed in those cases where n1 and n2 responses were also present. In 7 cases a small amplitude negative wave was observed in layer III, close to layer II at a latency of 5-8 ms (n4; fig. 3.2).

The most prominent feature of the evoked response induced by subiculum stimulation, however, was a sequence of events involving multiple layers. Based on two prominent events in layer V and layer III we refer to this sequence of events as the early population spike-wave complex (early PS-W complex). In deep entorhinal layers this response consisted of a sharp negative deflection, called here layer V population spike (labeled ‘ps’ in fig. 3.2; pop. spike peak latency 11.3 ± 0.6 ms, n=10/15). Following the layer V population spike a slow negative wave developed in layer III (labeled ‘w3’ in fig. 3.2). The latency of the maximal negativity of this layer III wave with respect to the peak of the layer V population spike was 5.0 ± 0.3 ms. At low intensity stimulation, a small amplitude, broad wave was present in layer V, but no population spike was evoked and also the layer III wave was absent (fig. 3.3).
A long latency evoked response was observed in 8 cases, which was usually only seen after the test stimulus in a paired pulse protocol. In 6 of these experiments the response had similar characteristics as the early PS-W complex, i.e. a population spike in deep layers and a negative wave in layer III (fig. 3.4), hence we designated it delayed population spike-wave complex (delayed PS-W complex). The latency of the delayed layer V population spike was in the range of 25-35 ms.

During a high frequency stimulation train (250 Hz) the early superficial layer components n1 and n2 decreased slightly in amplitude, but were still present (not shown).
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**Figure 3.2** Subiculum evoked responses in the entorhinal cortex. A. Typical example of laminar profiles of field potentials responses (average of 4 sweeps) in the entorhinal cortex evoked by subiculum stimulation (indicated by star). Early negative components in superficial layers (n1-n4) and the population spike (ps) and layer III wave (w3) components of the PS-W complex are indicated. Additional shading is used for the n1 (vertical lines) and n2 (gray) components. Scheme of the layers of the entorhinal cortex on the left indicates the location of the recording sites, as reconstructed from histology shown in figure 3.1B. B. The same field potential responses shown in A are illustrated in a contour plot at 0.1 mV increments. Negative potentials are drawn as black lines; positive potentials are drawn as dashed lines. The zero-level contour is not plotted. C. Details of the *early PS-W complex*. Note that only recording sites 4-13 are shown, covering layer VI-III as indicated by the scheme in D. The start of the population spike (ps) at each recording site (indicated by arrows) can be clearly recognized in the first time derivative (see inset for examples for recordings sites 10 and 11; dashed line represents raw signal, thick line represents first time derivative). The population spike component apparently propagated towards superficial layers and was followed by a negative wave in layer III (w3). The vertical line marks the start of the population spike at recording site 11. D. Field potential and current source density depth profiles of population spike (ps) and the layer III wave (w3) components constructed at the peak in layer V and layer III respectively (latencies are indicated between brackets). Relevant sinks and sources are shaded gray.

This is compatible with the interpretation that the early superficial layer events (*i.e.* n1 and n2) were the result of antidromic activation of perforant path fibers that pass through the subiculum (see Discussion). The subsequent components (*n3* and *n4*) possibly reflect the activation of local neurons through antidromically activated axon collaterals or the activation of direct hippocampal to entorhinal superficial layer projections (see Discussion). Both the *early* and *delayed PS-W complex* were considered to reflect the process of deep-to-superficial layer communication, therefore we describe these responses in detail below.

**Generation of the delayed PS-W complex involves the tri-synaptic pathway of the hippocampal formation**

The *delayed PS-W complex* was evoked optimally using a paired pulse protocol with intervals ranging from 50-100 ms (fig. 3.4A). It was never observed at intervals ≤25 ms and only rarely seen at intervals ≥150 ms. In most cases, the *delayed PS-W complex* appeared at higher stimulus intensities than the *early PS-W complex*. However, in one case a *delayed PS-W complex* could be observed without prior *early PS-W complex*. Therefore, we excluded the possibility that the *delayed PS-W complex* arose from local rebound activity in the entorhinal cortex as a result of the prior *early PS-W complex*. Occasionally, a full *delayed PS-W complex* did not develop, but rather a small negative wave was present in deep layers without subsequent superficial layer activation (see for example fig. 3.4A, 50 and 100 ms intervals). In most cases, both population spike and layer III wave of a fully developed *delayed PS-W complex* had large amplitudes, although in some cases, the deep layer population spike of the *delayed PS-W complex* was relatively small, compared with the layer III wave.
Figure 3.3 Entorhinal responses evoked at different stimulus intensities. A. Comparison of subiculum evoked potentials in the entorhinal cortex after low (left, 200 μA) and high (right, 500 μA) intensity stimulation. At low intensity stimulation a negative wave without population spike is present in deep layers and no layer III wave is present. Deep layer responses after low intensity stimulation are also superimposed onto high-intensity evoked responses (dashed lines). B. Field potential depth profiles at the times indicated in A. For the high intensity stimulation the population spike (ps) and layer III wave (w3) are superimposed.

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Figure 3.4 Subiculum evoked delayed PS-W complex. A. Averaged evoked field potentials in the entorhinal cortex evoked by test stimuli in subiculum at variable intervals (50-100 ms) after a conditioning stimulus (stim 1). At all intervals an early PS-W complex was evoked (ps: layer V population spike; w3: layer III negative wave), but only at 75 ms interval a full delayed PS-W complex could be observed (indicated by box). Note the presence of an incomplete delayed PS-W (black arrows) mostly limited to deep layers at 50 ms and 100 ms intervals. Only 4 of the 16 recording sites are shown. B. Simultaneous recording in hippocampal formation and entorhinal cortex demonstrated a correlation between the tri-synaptically evoked CA1 response and the delayed PS-W complex. Two individual sweeps from one experiment after stimulation with identical intensity are shown. Subiculum stimulation evoked an early PS-W complex in the entorhinal cortex and also activated the tri-synaptic pathway of the hippocampal formation. A delayed PS-W complex was present in the entorhinal cortex if also a population spike occurred in CA1 (right), but not in the absence of a CA1 population spike (left). Note also the presence of an antidromic population spike in CA1. Only a selection of the available recording sites is shown. C. Summary of 14 experiments in which recordings were performed in both hippocampal formation and entorhinal cortex. The table shows the number of experiments with a certain amount of activation of CA1 through the tri-synaptic pathway (i.e. no response, small excitatory post-synaptic potential (EPSP) only, small population spike or large population spike) and the correlation with the size of the delayed PS-W complex in the entorhinal cortex (no delayed PS-W complex, small delayed PS-W complex limited to deep layers or full delayed PS-W complex).
As we suspected that stimulation in the subiculum also activates passing fibers of the perforant path, we tested if hippocampal activation could underlie the generation of the delayed PS-W complex. As shown in figure 3.4B, subiculum stimulation resulted in responses that are usually associated with activation of the hippocampal tri-synaptic pathway after classic perforant path stimulation in the angular bundle. Thus, in the dentate gyrus a large
positive potential was observed in the hilus and granule cell layer, which reversed in the molecular layer. This response was associated with a current sink in the molecular layer and current source in the granule cell layer (not shown). Usually, a negative-going spike transient, representing the simultaneous firing of granule cells, was superimposed onto the positive wave. In addition, a longer latency (peak latency in the range of 17-20 ms) negative potential and an associated current sink were observed in stratum radiatum of CA1, and a corresponding current source was present in CA1 pyramidal layer (not shown). Occasionally, CA1 neurons fired action potentials simultaneously as evidenced by a population spike in the pyramidal cell layer. Simultaneous recordings in the hippocampal formation and MEA in 14 experiments indicated a correlation between the delayed PS-W complex and the tri-synaptically evoked CA1 response. In general, the delayed PS-W complex was only present in those cases in which a CA1 population spike could be observed as well (fig. 3.4B,C). Occasionally, the CA1 population spike was small and in those cases the delayed PS-W complex was also small and usually limited to the deep layers.

If the delayed PS-W complex was indeed the result of activation of the hippocampal tri-synaptic pathway, we assumed that stimulation at any other location within this pathway would also result in a PS-W complex in the entorhinal cortex. Indeed, stimulation of the Schaffer collaterals in CA3 also evoked a population spike in layer V, followed by a negative wave in layer III (labeled ‘ps’ and ‘w3’ respectively in fig. 3.5A). The latency of the Schaffer simulation induced PS-W complex was in between those of the early and delayed PS-W complex after subiculum stimulation (pop. spike peak latency 18.3 ± 1.0 ms, range 15.1-20.9 ms, n=6). Similar to the delayed PS-W complex, the Schaffer stimulation induced PS-W complex in some cases had a small layer V population spike, followed by a large superficial layer III wave. Note also in figure 3.5 that the Schaffer stimulation evoked PS-W complex resulted in a positive-negative biphasic wave in layer I (labeled ‘wl’ in fig. 3.5).

These results indicate that stimulation in the subiculum results in indirect activation of the hippocampal output structures via tri-synaptic pathway, which eventually leads to a delayed PS-W complex in the entorhinal cortex. We therefore considered all occurrences of a PS-W complex in the entorhinal cortex as reflecting the activation of hippocampal outputs.

**Detailed description of the PS-W complex**

*Laminar profiles and current source density analysis*

The population spike of the early PS-W complex had maximal amplitude in layer V. Going from layer V in the direction of more superficial layers, the population spike diminished in amplitude and gradually changed from a negative-going deflection to a biphasic wave and finally became positive-going close to layer II (fig. 3.2C). The population spike appeared to propagate from deep to superficial layers, since the peak latency of the population spike was shortest in deep layers and gradually increased in layer III (corresponding to a
propagation speed of about 0.2 m/s), at the same time the population spike gradually became broader.

In layer V, the population spike usually appeared on top of a negative wave of smaller amplitude and longer duration. In many cases a sudden change in the slope of the layer V evoked potential marked the start of the population spike (fig. 3.2C, arrows). This was particularly clear in the derivative of the evoked response (fig. 3.2C, inset). The latency of the start of the population spike increased from layer V towards layer III, similar to the latency to peak. At low intensity stimulation only the underlying negative wave was present in deep layers (fig. 3.3A), which had a similar depth profile as the population spike in layer V (fig. 3.3B). Current source density analysis showed that the layer V population spike corresponded to a sharp sink in layer V and sources in layer VI and III (fig. 3.2D). As was the case in the field potential recordings, the population spike sink propagated from layer V towards layer III.

The layer III wave of the PS-W complex after subiculum stimulation had its maximal amplitude in layer III and reversed close at the border with layer II (fig. 3.2C,D). Usually the negative wave continued into layer V and reversed midway the deep layers. The layer III

![Figure 3.5](image)

**Figure 3.5** Schaffer collateral stimulation evoked responses in the entorhinal cortex. **A.** Laminar field potential profile of the PS-W complex in the entorhinal cortex evoked by Schaffer-collateral stimulation. Both the layer V population spike (ps) and the layer III negative wave (w3) were present. Note the longer onset latency as compared to the early PS-W complex evoked by subiculum stimulation. Also note that in layer I an additional positive-negative wave (w1) was present. The location of the recording sites is shown in the scheme on the left, as reconstructed from histology. **B.** Field potential and current source density depth profiles constructed at the peaks (latency indicated between brackets) of the population spike (ps), layer III wave (w3) and layer I negativity (w1). Relevant sinks and sources are shaded gray.
Figure 3.6 Short-term dynamics of the subiculum evoked early PS-W complex. A. Left: response after conditional stimulus (stim 1). Right: responses after test stimulus (stim 2) at varying intervals after the conditional stimulus. Note the appearance of an additional layer I negative-going wave at 25-100 ms inter-pulse intervals. B. Quantification of the paired pulse ratio (peak amplitude after stim 2 / peak amplitude after stim 1) as function of the inter-pulse interval. For the layer V population spike (top) the amplitude was determined with reference to the start of the population spike (see inset), for the layer III wave (bottom) the amplitude was determined with reference to zero potential (see inset). C. Top: The amplitude of the layer V population spike (black dots) and layer III wave (circles) after the conditioning stimulus (stim 1) and test stimulus (stim 2) are linearly related. For this analysis all data points at 50, 75 and 100 ms inter-pulse intervals were grouped. Lines indicate best linear fit. Bottom: Relation between the amplitude after the conditioning stimulus (p1) and the paired pulse ratio. For both layer V population spike (black dots) and layer III wave (circles) the paired pulse ratio increases with smaller amplitude after stim 1.
wave was never observed without a preceding deep layer population spike. In contrast with the Schaffer stimulation evoked PS-W complex, there were no cases in which a relatively small layer V population spike was associated with a large layer III wave. Current source density analysis showed that the layer III wave corresponded to a sink in layer III close to layer V and a source in layer II and I and in some cases a source in layer V, just deep to the sink in layer III (fig. 3.2D). We should note that in the cases that a $n4$ component was clearly present, this had a similarly located sink in layer III as the $w3$ component. This raises the possibility that the $n4$ and $w3$ components may represent at least partly the same synaptic event, although the $w3$ event could be evoked without an obvious preceding $n4$ component.

Comparison of the early PS-W complex evoked by subiculum stimulation (fig. 3.2C,D) and the PS-W complex evoked by Schaffer collateral stimulation (fig. 3.5A,B) showed that the laminar field potential profiles and CSDs of both the layer V population spike and the layer III wave were similar.

**Short-term dynamics of early PS-W complex**

A striking feature of subiculum-evoked early PS-W complex in the entorhinal cortex was that both the layer V population spike as well as the layer III wave had a larger amplitude and a shorter latency to peak after the test stimulus in a paired pulse protocol. Varying the interval between the two stimuli showed that facilitation was optimal at 25-100 ms intervals for both events (moderate stimulation intensity; fig 3.6A,B), however there was a large variation among experiments. Since paired pulse ratios at 50, 75 and 100 ms inter-pulse intervals were similar, these were grouped. As shown in figure 3.6C (top), for this group the amplitude after the second stimulus was related linearly to the amplitude after the first stimulus. It was possible for the second stimulus to evoke a response, without a detectable response after the first stimulus (i.e. the fitted lines in fig. 3.6C do not pass through the origin). Usually, this happened at low stimulation intensity. As a consequence, the paired pulse ratio increased with smaller amplitude responses after the first stimulus (fig. 3.6C, bottom). This explained the large variation in paired pulse ratio in figure 3.6B, since between experiments there was variation in the actual amplitude of the first response. Regardless of this variation, in all cases the paired pulse ratio was larger than unity.

As illustrated in figure 3.6A (black arrowhead), an additional negative going wave was evoked in layer I, particularly at 25-100 ms intervals. This layer I negative going wave had similar laminar profiles and associated CSDs (not shown) as the layer I wave that was part of the Schaffer collateral stimulation evoked PS-W complex (see fig. 3.5A,B).

**Single unit responses**

To further characterize the PS-W complex, evoked responses of single units in the entorhinal cortex were recorded after subiculum stimulation. Of 169 cells (layers V-VI: n=55;
Figure 3.7 Subiculum evoked responses of entorhinal deep layer neurons. A. Summary of single unit responses of deep layer neurons evoked by paired pulse stimulation of subiculum. Stim 1: conditioning stimulus; stim 2: test stimulus at 100 ms interval. Plots were created by averaging the peri-stimulus time histograms (PSTH) of all responsive cells. For those cases in which a clear field potential was present, units were classified as occurring during the population spike of the early PS-W complex (dark gray bars) or the delayed PS-W complex (light gray bars). Black bars represent the remaining cases. B. Example responses of two deep layer neurons after a conditioning stimulus at 100 ms interval. Both the PSTH (gray bars) and averaged evoked potential (line) are shown. Both cells fired during the population spike component of either the early (right) or delayed (left) PS-W complex. C. Example of a layer V pyramidal neuron that responded to subiculum stimulation (responses of this neuron are shown in B, right). The cell was labeled with neurobiotin (left) and reconstructed with NeuroLucida (right). D. Example of inhibitory responses of a deep layer neuron, as evidenced by a transient decrease of unit firing probability (indicated by arrow) after subiculum stimulation (stimulus artifacts indicated by stars). An overlay of 18 traces is shown, each high-pass filtered at 300 Hz. This particular cell did not show excitatory responses after stimulation.

layer III: n=107; layer II: n=7) it was confirmed that they were located in entorhinal cortex. From this group, 64 cells showed a clear increase in firing rate upon subiculum stimulation. Entorhinal deep layer neurons that responded to subiculum stimulation (n=32) did not show evoked responses at very short latencies. As shown in the histogram in figure 3.7A, most units fired around 10 ms (range 5-15 ms). Those cases in which a clear field potential could be recognized, along with distinct unit firing, deep layer neurons responded in
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Figure 3.8 Subiculum evoked responses of entorhinal superficial layer neurons. A. Summary of single unit responses of superficial layer neurons evoked by paired pulse stimulation of subiculum. stim 1: conditioning stimulus; stim 2: test stimulus at 100 ms interval. Plots were created by averaging the peri-stimulus time histograms (PSTH) of all responsive cells. White bars represent presumed antidromic spikes. For those cases in which a clear field potential was present, units were classified as occurring during layer III wave of the early PS-W complex (dark gray bars) or the delayed PS-W complex (light gray bars). Black bars represent the remaining units. B. Example responses of one layer III pyramidal neuron. The PSTH (gray bars) and averaged evoked potential (line) after a test stimulus (left) and a conditioning stimulus at 100 ms interval (right) are shown. Note that this neuron fired at early latencies and during the layer III wave of the early and delayed PS-W complex. This cell was identified as a layer III pyramidal neuron after labeling with neurobiotin (C, left) and reconstructing with Neurolucida (C, right). D. The example neuron shown in B and C, also demonstrated a transient decrease of firing (indicated by arrow) upon subiculum stimulation (stimulus artifacts indicated by stars). An overlay of 9 traces is shown, each high-pass filtered at 300 Hz.

association with the population spike component of either the early (dark gray bars in fig. 3.7A, see example fig. 3.7B, right) or delayed PS-W complex (light gray bars in fig. 3.7A; see example in fig. 3.7B, left). It is clear from figure 3.7A that the firing probability of deep layer neurons during either early or delayed PS-W complex was increased after a test stimulus (stim 2) in a paired pulse protocol (100 ms inter-pulse interval). Some units did not relate to a (recognizable) field potential component (indicated by black bars in fig. 7A) and many of
these fired just before or after the peak in the histogram corresponding to the population spike. These units probably corresponded to cells that fired asynchronously from neighboring cells and thus did not contribute to the field population spike. The responsive cells in layer V that were labeled with neurobiotin were exclusively pyramidal-shaped neurons (n=5, see example fig. 2.7C), whereas responsive neurons in layer VI displayed morphological characteristics of multipolar neurons (n=3).

Superficial layer neurons (layer III: n=28; layer II: n=3) responded at different latencies after subiculum stimulation. In figure 8A several peaks can be observed in the histogram at which superficial layer neurons preferably responded. Some units had very short latencies (1-4 ms latency, n=7) and showed very little jitter in time, corresponding to antidromic activation (white bars in fig. 3.8A, see also example in fig. 3.8B). Four of these cells were labeled with neurobiotin and, after reconstruction, appeared to be a layer II stellate neuron, two layer III pyramidal neurons and a layer III multipolar neuron.

Several superficial layer units responded at latencies, which would correspond to the early (9-20 ms) or delayed (>25 ms) PS-W complex in the local field potential (n=16, dark gray bars in fig. 3.8A). If a clear layer III wave could be recognized in the local field potential these units usually appeared to respond during the descending part of the wave (fig. 3.8B). Some units increased their firing rate during the trough of the wave (i.e. see example fig. 3.8B), but units did not fire during the ascending part of the wave. Note in figure 3.8A, that the firing probability of superficial layer units was increased after a test stimulus (stim 2) that followed a conditioning stimulus (stim 1) at 100 ms. Neurobiotin labeled cells belonging to this group included three layer III pyramidal neurons (fig. 3.8C) and one layer III multipolar neuron.

A relatively large proportion of units responded at latencies between 4-8 ms (n=9; second peak in fig. 3.8A), which could not be classified as antidromic and also did not correspond to the layer III wave in the local field potential. In some cases, these units fired during a small field negativity, possibly corresponding to the n4 event in the field potential described above. In contrast to unit responses during the PS-W complex, firing probability was not enhanced in a paired pulse protocol (fig. 3.8A). Five cells of this group were labeled with neurobiotin and were located in layer III; four of these cells displayed characteristics of pyramidal neurons and one of a multipolar neuron.

At least 28 units were found that showed a clear inhibition of firing after subiculum stimulation (figs. 3.7D, 3.8D). For units, which had relatively high basal firing rates, reduction of firing was already clear after the first pulse in a paired pulse protocol, however inhibition was most pronounced after the second pulse (100 ms interval). Typically, the reduced firing lasted 200-600 ms, but in a few cases it lasted longer than 1000 ms. Both cells in deep layers (n=7; fig. 3.7D) and superficial layers (layer III: n=19; layer II: n=1; fig. 3.8D) could show inhibitory responses. It was possible to find inhibitory responses both in
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Figure 3.9 Effect of CNQX applied iontophoretically to deep entorhinal layers on responses evoked by subiculum (A) or local entorhinal (B) stimulation. pre: before CNQX application, cnqx: after CNQX application, post: after wash-out of CNQX. Note the disappearance of the responses in deep layers and the layer III wave after subiculum stimulation, but not other superficial layer responses or the evoked responses after local entorhinal stimulation. C. Nissl-stained sagittal section of the entorhinal cortex showing the location of CNQX application (arrow). Lamina dissecans is drawn as a dashed line.

combination as well as in the absence of excitatory responses (see examples in fig. 3.7D and 3.8).

**PS-W complex reflects deep-to-superficial layer communication**

To test the hypothesis that the layer III wave is the result of deep-to-superficial layer communication, CNQX was applied to the deep layers (fig. 3.9C) to block the layer V population spike evoked by subiculum or Schaffer collateral stimulation in three experiments. Both deep and superficial layer responses during the PS-W complex evoked by subiculum stimulation were abolished after CNQX application (fig. 3.9A). In all cases early superficial layer components n1-n3 (fig. 3.9A) were not affected. Also we were confident that in at least one case superficial layer responses evoked by local entorhinal stimulation were not affected (fig. 3.9B), showing that CNQX had not diffused into superficial layers. This result suggests that the layer III wave of the PS-W complex requires the prior activation of layer V, and thus that the PS-W complex reflects deep-to-superficial layer communication.
DISCUSSION

The main result of the present study, in short, is that 1) stimulation of hippocampal formation output results in a population spike-wave complex in the entorhinal cortex, which reflects sequentially activation of deep and superficial layers; and that 2) activation of the superficial entorhinal layers is the result of deep-to-superficial layer communication.

The experimental approach followed in this study comprised a combination of simultaneous multi-electrode recordings to construct depth potential and current-source density (CSD) profiles along with single unit recordings of morphologically identified cells. This was performed \textit{in vivo} in the intact, although anaesthetized, rat brain in order to obtain information about both the local circuitry of the entorhinal cortex and the regional circuitry of the hippocampal-entorhinal cortex complex. This kind of information cannot be obtained directly from \textit{in vitro} studies.

**Subiculum stimulation – what is stimulated?**

In this study electrical stimulation of the dorsal subiculum was used as a means to activate hippocampal-entorhinal pathway and to probe inter-laminar interactions in the entorhinal cortex. The subiculum-entorhinal cortex projection is topographically organized, such that the dorsal (septal) part of subiculum projects to a dorso-lateral strip of entorhinal cortex bordering the rhinal sulcus (chapter 2). Our electrophysiological results are consistent with this topography, since the largest responses were found in the dorsal part of MEA (close to the rhinal sulcus) after stimulation of the dorsal subiculum. Although our aim was to stimulate the subicular efferent pathway to the entorhinal cortex, we realized that the responses in the entorhinal cortex were also to some extent due to the activation of axons that terminate in or pass through the subiculum. For example, CA1 neurons that project to subiculum (Amaral et al., 1991) were antidromically activated as evidenced by a short latency population spike in the pyramidal cell layer of CA1 (see fig. 3.4B). Since both CA1 and subiculum project to the entorhinal cortex (chapter 2. Köhler, 1985, Tamamaki and Nojyo, 1995, van Groen and Wyss, 1990), it is possible that antidromic stimulation of the CA1-subiculum pathway via axon collaterals also results in orthodromic activation of the CA1-entorhinal cortex pathway (fig. 3.10). Therefore, we refer to subiculum stimulation as ‘activating hippocampal output’, rather than claiming that the evoked responses in the entorhinal cortex are solely due to the subiculum-entorhinal cortex projection. Subiculum stimulation also activated passing fibers of the perforant path, which originate in layer II and III of the entorhinal cortex and project to all sub-fields of the hippocampal formation (fig. 3.10). This was evidenced by 1) short latency, presumably antidromic field potentials (events \(n_1\), \(n_2\)) in layers II/III, which follow 250 Hz stimulation, 2) short latency single unit responses in superficial layers which displayed minimal time jitter and 3) orthodromic responses in the dentate gyrus. Furthermore, if the \(n_1\) and \(n_2\) events were mediated by the sparse direct
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Figure 3.10. Summary scheme of hippocampal-entorhinal connections and intra-entorhinal circuitry involved in the generation of the different components of the evoked responses observed in MEA. 1: Subiculum stimulation activates entorhinal deep layers (corresponding to the layer V population spike) by direct subiculum-entorhinal cortex projections and possibly by CA1-entorhinal cortex efferents (black dotted arrow). 2: Activity of deep layer pyramidal neurons and interneurons is conveyed to superficial layers (corresponding to w3 wave). 3: Subiculum stimulation results in antidromic activation of layer II and III neurons due to stimulation of passing fibers of the perforant path (corresponding to components n1 and n2), and (4) in activation of associational connections in superficial layers (corresponding to component n4). 5, 6: Stimulation of passing fibers of the perforant path results also in a sequential activation of hippocampal subfields via the trisynaptic pathway, eventually leading to a secondary activation of hippocampal output, which elicits a delayed PS-W complex in MEA.

hippocampal to entorhinal superficial layer projections (chapter 2, Köhler, 1985), we would expect similar evoked potentials at a slightly longer latency after Schaffer collateral stimulation, but this was not the case.

Taking into account the mixed nature of subiculum stimulation and the anatomical connections described in the hippocampal-entorhinal system, our interpretation of the evoked field potentials and single unit responses in the entorhinal cortex is as depicted in figure 10.

Nature of deep layer population spike

We found that stimulation of hippocampal output excited pyramidal neurons in layer V, which appeared in the field potential as a population spike, and was associated with a current sink in CSD analysis. The population spike was superimposed onto a small negative wave, which likely represent a monosynaptic population EPSP. A similar potential in deep entorhinal layers was found following long-latency hippocampal responses evoked by lateral olfactory tract stimulation in the whole brain guinea pig preparation (Biella and de Curtis, 2000). Indeed, anatomical studies have shown that the projections from the hippocampal formation terminate predominantly in layer V of the entorhinal cortex (chapter 2, Köhler, 1985, van Groen and Wyss, 1990). These projections mainly involved asymmetrical excitatory synapses onto spine-bearing, presumably pyramidal neurons and onto dendritic shafts of presumed inhibitory neurons (van Haeften et al., 1995). Also, intracellular recordings in vivo and in vitro showed excitatory responses of deep layer entorhinal neurons upon subiculum stimulation (Finch et al., 1986, Jones, 1987).
Our laminar field potential recordings showed that the population spike evoked in layer V apparently propagated to superficial layers. This probably reflects invasion of action potentials in the long apical dendrites of layer V pyramidal neurons that extend into superficial layers. Ample evidence exists that dendritic backpropagation of action potentials occurs in neocortical areas (Buzsáki and Kandel, 1998, Finch et al., 1986, Jones, 1987, Markram et al., 1995, Stuart et al., 1997, Stuart and Sakmann, 1994) and hippocampal area CA1 (Kloosterman et al., 2001, Miyakawa and Kato, 1986, Richardson et al., 1987, Spruston et al., 1995) and it is not unlikely that the apical dendrites of entorhinal layer V neurons also possess the mechanisms that support generation of action potentials. Interaction between back-propagating action potentials and synaptic inputs may result in modification of the active synapses, depending on the timing between the dendritic action potential and the synaptic input (Markram et al., 1997). A second consequence of dendritic back-propagation of action potentials may be to increase the influence of distal dendritic inputs and thereby associating inputs arriving at different cortical layers (Larkum et al., 1999).

Strikingly, deep layer responses showed only frequency-dependent facilitation and no depression. The absence of frequency-dependent depression may suggest that feedback inhibition is not prominent in deep layers of the entorhinal cortex at the population level. Nevertheless, our unit recordings showed that some cells displayed increases in firing rate followed by long lasting inhibition, which could be mediated by inhibitory feedback connections. Previously, in the entorhinal cortex inhibition has been suggested to be exerted predominantly through feed-forward connections (Finch et al., 1988). This has been corroborated by the demonstration of a substantial excitatory projection onto presumed deep layer interneurons (van Haeften et al., 1995). We did not explore the mechanism responsible for the phenomenon of frequency-dependent facilitation. However, recent studies showed that presynaptic metabotropic glutamate receptors may increase glutamate release (Evans et al., 2000) and decrease release of GABA onto layer V pyramidal neurons in vitro (Woodhall et al., 2001). Such a combination of effects could contribute to the observed paired pulse facilitation of the field potential responses.

Nature of superficial layer responses

Several processes may contribute to the second component of the early PS-W complex, i.e. the layer III wave \((w3)\) that followed the population spike in layer V. A first possibility is that the \(n4\) and \(w3\) components, which both have similar laminar CSD profiles, are part of a single monosynaptic EPSP, only temporally interrupted by sinks/sources associated with the layer V population spike. However, the \(w3\) event could also be found without preceding \(n4\) component, this was particularly clear for the delayed PS-W complex and for SCHAF stimulation evoked PS-W complex. In addition, whereas the \(w3\) component showed prominent paired pulse facilitation, this was not obvious for the \(n4\) component. This suggests that the \(n4\) and \(w3\) component may in fact represent separate responses. The \(n4\) component may represent
a subiculum evoked monosynaptic EPSP in layer III, direct projections from subiculum to superficial layers exist, but these are rather sparse in comparison with projections to deep layers (chapter 2, Köhler, 1985). In this respect, it is remarkable that many superficial layer neurons appear to fire at the time of the $n_4$ component (i.e. 5-8 ms latency, fig. 3.8A). If a direct subiculum-superficial layer projection would have contributed to the $n_4$ responses, it could be expected that just prior to the delayed PS-W complex and SCHAF evoked PS-W complex an event similar to the $n_4$ component would be observed, but this was not the case. Thus the alternative explanation is more likely, namely that the $n_4$ component is due to local associational connections of layer II and III neurons, which are antidromically activated. Such associational connections have been demonstrated both anatomically (Dolorfo and Amaral, 1998b) and electrophysiologically (Dhillon & Jones, 2000; Biella et al., 2002).

A second possibility is that the $w_3$ component may correspond to an active source in deep layers and an associated passive sink located more superficially, due to synchronous GABA-mediated IPSPs or spike after-hyperpolarization in layer V neurons. Indeed, a few deep layer neurons were recorded that showed reduced firing after stimulation. Whether this is mediated by feed-forward or feed-back inhibition cannot be decided on the basis of the present experiments. At both the population and single unit level, however, paired pulse depression was not observed, suggesting that feed-back inhibition did not contribute much to observed responses. The contribution of an active source in deep layers cannot explain the $w_3$ event completely, since 1. the sink in layer III was always stronger than the source in deep layers, 2. the $w_3$-sink was associated with a source more superficially in layers I/II and 3. superficial layer neurons fired during the $w_3$ component, indicating that the sink in superficial layers was not merely passive.

A third possibility is that the $w_3$ component is di-synaptically generated via synapses in deep layers. This interpretation is supported by the demonstration that CNQX applied to deep layers abolished the $w_3$ component. Furthermore, single unit firing of layer III neurons coincided with the $w_3$ component and both single unit firing and the $w_3$-sink were facilitated in a paired pulse protocol. The apparent delay of the layer III wave with respect to the layer V population spike is compatible with this interpretation.

Thus, we conclude that at least part of the $w_3$ event is due to an activation of the projection from deep layer neurons to superficial layers.

**Nature of intrinsic deep-to-superficial entorhinal circuitry**

The fact that axons of entorhinal deep layer neurons project to superficial layers was already noted by Cajal (Ramón y Cajal, 1955, pages 689-698) and it was recently further demonstrated using anterograde tracing techniques (Dolorfo and Amaral, 1998b, Köhler, 1986, van Haeften et al., 2003). A motivation to initiate the present study was the recent investigation of the synaptic interactions between neurons in entorhinal deep and superficial layers (van Haeften et al., 2003). How can we connect our electrophysiological results to
these and other relevant anatomical data? A large part of the deep-to-superficial layer projection terminates in layer III and is excitatory (van Haeften et al., 2003), in agreement with the occurrence of the \( w3 \) sink and the associated firing of neurons in layer III. These projections were not found to terminate on apical dendrites of deep layer neurons that extend into superficial layers, suggesting that EPSPs in these dendrites are less likely to contribute to the observed \( w3 \) response. Almost 50% of the projection from deep to superficial layers consists of asymmetrical synaptic contacts on presumed inhibitory interneurons (van Haeften et al., 2003), constituting a prominent feed-forward inhibitory projection. This pathway may mediate the long-lasting reduction of firing rate observed for some superficial layer neurons. A relatively small portion of the deep-to-superficial layer projection consists of symmetrical synaptic contacts (assumed inhibitory) onto supposed inhibitory interneurons (van Haeften et al., 2003), possibly resulting in reduced firing of these interneurons and disinhibition of superficial layer principal neurons. This disinhibition could contribute to the observed frequency dependent facilitation of the \( w3 \) component.

In the guinea pig, dorsal psalterium stimulation (mainly containing fibers originating in ipsi- and contralateral presubiculum (Bartesaghi, 1985)) resulted in entorhinal responses that depended on hippocampal-entorhinal connections and which resembled our \( PS-W \) complex (Bartesaghi et al., 1989). Based on the increased latency of superficial layer responses compared to deep layer responses Bartesaghi et al. (1989) hypothesized that the former were evoked by intracortical connections, what is in agreement with our present findings.

In some \textit{in vitro} studies electrical stimulation of deep layers was used to study synaptic inputs of superficial layers (Bartesaghi et al., 1989, Biella et al., 2002, Gloveli et al., 1997a, Gloveli et al., 1997b, Jones, 1994, Richter et al., 1999), but this likely caused a mixed activation of axons originating from deep layer neurons and axons from extrinsic sources which after leaving the white matter pass through deep layers to terminate in superficial layers. Nevertheless, deep-to-superficial layer communication has been observed in hippocampal-entorhinal slices under conditions of enhanced excitability (Dickson and Alonso, 1997, Iijima et al., 1996, Jones and Lambert, 1990, Lopantsev and Avoli, 1998, Stewart, 1999).

During slow-wave sleep the activity of deep layer entorhinal neurons is correlated with events called sharp waves in area CA1 (Chrobak and Buzsaki, 1994). In the same study relatively few layer III neurons and no layer II neurons showed sharp-wave related activity, and therefore the authors suggested that during slow-wave sleep practically no communication takes place between deep and superficial layers. Nevertheless, we show here that this communication can take place even in anesthetized animals.

\textbf{Functional relevance of deep-to-superficial layer communication in the entorhinal cortex}

A vast amount of evidence indicates that the entorhinal cortex and hippocampal formation are required for the formation of new memories of facts and events. Multi-modal
sensory information reaches the hippocampal formation through neurons in layer II and III of the entorhinal cortex. Hippocampal output is distributed to the neocortex by way of the output projections from area CA1 and subiculum to the deep layers of the entorhinal cortex. It has been suggested that the reciprocal connections between the entorhinal cortex and the hippocampal formation constitutes a so-called re-entrant circuitry. The projection from deep to superficial layers is crucial in this respect, since it may provide a substrate for the interaction between hippocampal output and hippocampal input pathways. Entorhinal dependent re-entrance of hippocampal output back into the dentate gyrus has been demonstrated in anesthetized rats (Deadwyler et al., 1975, Wu et al., 1998). Re-entrance into CA1 or subiculum via the direct entorhinal layer III projections to these areas, has also been found recently (Bartesaghi and Gessi, 2003). Re-entrance of information may eventually lead to reverberation of activity in a closed loop, providing a means by which information is dynamically stored for a short time. This temporarily held information could then be compared to new sensory inputs arriving in superficial layers of entorhinal cortex, possibly forming a novelty detection network (Naber et al., 2000). In addition, reverberation could provide the network with repeated exposures to the same memory traces and thereby facilitating the synaptic modifications necessary for storing the trace (Wittenberg and Tsien, 2002).