Stimulation of the Parasubiculum Modulates Entorhinal Cortex Responses to Piriform Cortex Inputs In Vivo

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Submitted 12 January 2004; accepted in final form 16 March 2004

INTRODUCTION

Multi-modal sensory information carried by inputs from the piriform, perirhinal, and postrhinal cortices (Amaral and Witter 1995; Burwell and Amaral 1998) converges within the superficial layers of the entorhinal cortex. Neurons in layer II of the entorhinal cortex, in turn, provide the hippocampus with the majority of its cortical sensory input through the perforant path projection to the dentate gyrus and CA3 region and a smaller projection from layer III reaches the CA1 region and subiculum (Amaral and Witter 1995; Witter et al. 1989). The factors that govern synaptic integration and temporal firing patterns in entorhinal cortex projection neurons are therefore likely to contribute to the information processing and mnemonic functions of the hippocampal formation.

The subiculum and the hippocampal CA1 region are well known to project to the deep layers of the entorhinal cortex (Amaral and Witter 1995; Amaral et al. 1991), but the presubiculum and parasubiculum target the superficial layers and may therefore modulate responses of entorhinal cortex projection neurons to extrahippocampal cortical afferents. The presubiculum projects to layers I and III of the medial entorhinal cortex with the majority of synaptic contacts on dendrites of layer III neurons (Caballeró-Bleda and Witter 1994; Köhler 1984; van Groen and Wyss 1990). The parasubiculum, however, targets layer II of both the medial and lateral entorhinal cortex (Caballeró-Bleda and Witter 1993, 1994; Köhler 1985; van Groen and Wyss 1990), the laterodorsal, anteroventral, and anterodorsal thalamic nuclei (Shibata 1993; van Groen and Wyss 1992, 1995), and the basolateral amygdala (van Groen and Wyss 1990). The manner in which the parasubiculum modulates entorhinal cortex responses to sensory inputs may therefore depend on afferents from subcortical structures as well as on changes in activity within the hippocampal formation.

Although excitatory projections from the parasubiculum to the entorhinal cortex provide an intriguing pathway for the re-entrance of activity back into the hippocampal circuit, the superficial layers of the entorhinal cortex are dominated by high levels of inhibition (Funahashi and Stewart 1998; Jones 1993–1995), and strong inhibitory responses have been observed in the few studies that have characterized parasubicular inputs to layer II electrophysiologically. Both AMPA and N-methyl-D-aspartate (NMDA) receptor-mediated excitatory postsynaptic potentials (EPSPs) are evoked in layer II neurons after stimulation of the parasubiculum in vitro (Jones 1990), but local inhibitory interneurons are also activated (Jones and Buhl 1993), and synaptic responses in principal neurons are predominantly inhibitory (Jones 1990). Stimulation of various sites within the hippocampus, subicular complex, and amygdala in vivo also evokes strong inhibitory responses in layer II cells (Colino and Fernandes de Molina 1986; Finch and Babb 1980; Finch et al. 1986, 1988). It is therefore not known if parasubicular inputs to the entorhinal cortex promote or inhibit entorhinal cortex responses to extrahippocampal cortical afferents.

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The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
The present study used field potential recording techniques to characterize synaptic responses evoked by parasubicular inputs to the layer II of the medial entorhinal cortex in awake, adult rats and to examine the role of the parasubiculum in modulating synaptic responses evoked by the large input from the piriform cortex (Burwell and Amaral 1998; Chapman and Racine 1997a,b; Chapman et al. 1998b). Because field potentials generated by this pathway have not been reported previously, current source density analysis in acute preparations was used to determine the synaptic origins of field potential responses in layer II of the entorhinal cortex. Tests conducted in awake, freely behaving animals indicate that the response is generated monosynaptically and suggest that both the parasubiculum and piriform cortex target a common population of layer II neurons. Paired-pulse stimulation was then used to determine the time courses of inhibitory and facilitatory mechanisms evoked by parasubiculum stimulation and to characterize the effect of parasubiculum stimulation on responses of the entorhinal cortex to subsequent activation of the piriform cortex.

METHODS

Acute recordings and CSD analysis

SURGERY. Male Long-Evans hooded rats (350–450 g; n = 10) were anesthetized with urethane (1.5 g/kg ip) and placed in a Kopf stereotaxic apparatus with bregma and lambda leveled within 200 μm. Anesthetized with urethan (1.5 g/kg ip) and placed in a Kopf stereotaxic (0.1-Hz to 5-kHz passband), amplified (A-M Systems, Model 2010D) and/or heating lamp. A bipolar stimulating electrode made from Teflon-coated stainless-steel wire (125 μm exposed tips separated by 0.8 mm) was lowered through a burr hole into the right parasubiculum (P, 8.1 mm; L, 3.8 mm; V, 5.0 mm relative to bregma). Monopolar field potential recordings were obtained using a tungsten electrode (50- to 80-μm tip diameter) placed in an oil-hydraulic micromanipulator (Narishige, Model 2200) and a stimulus isolation unit (A-M Systems, Model 2200). Pulses to the parasubiculum. Evoked (50 kHz), and a stimulus isolation unit (A-M Systems, Model 2200) were generated by inward and outward membrane currents, and the equation current source density analysis in acute preparations was used to determine the synaptic origins of field potential responses in layer II of the entorhinal cortex. Tests conducted in awake, freely behaving animals indicate that the response is generated monosynaptically and suggest that both the parasubiculum and piriform cortex target a common population of layer II neurons. Paired-pulse stimulation was then used to determine the time courses of inhibitory and facilitatory mechanisms evoked by parasubiculum stimulation and to characterize the effect of parasubiculum stimulation on responses of the entorhinal cortex to subsequent activation of the piriform cortex.

DEPTH PROFILE RECORDINGS. The amplitude of evoked responses was maximized by placing the recording electrode in the superficial layers of the entorhinal cortex 200 μm from the cortical surface and by adjusting the vertical position of the stimulating electrode to minimize current thresholds. Electrical stimuli were generated with either a pulse generator (AMPI, Master 8) or a computer D/A channel (50 kHz), and a stimulus isolation unit (A-M Systems, Model 2200) was used to deliver 0.1-ms biphasic constant current square-wave pulses to the parasubiculum. Evoked field potentials were analog filtered (0.1-Hz to 5-kHz passband), amplified (A-M Systems, Model 1700), and digitized at 10 or 20 kHz (16 bit) for storage on computer hard disk using the software package Experimenter’s Workbench (Datawave Tech.). A 30-min baseline period preceded experimental testing, and pulse intensity was then set to evoke responses that were 75% of maximal levels (300–800 μA) for subsequent recordings.

To localize synaptic currents that generate evoked responses, field potential recordings for current source density analysis were obtained at multiple depths in the entorhinal cortex. Responses were evoked by paired-pulse stimulation of the parasubiculum and, in most cases (10 of 12), were recorded at 31 depths as the recording electrode was retracted from the cortical surface in 50-μm steps. In two cases, recordings were obtained as the electrode was advanced to the cortical surface to avoid the possibility that the electrode track could shunt extracellular currents. Preliminary experiments demonstrated a strong facilitation using a 40-ms interpulse interval, and paired-pulse stimulation at this interval was used to better visualize responses. Ten responses were recorded at each depth using an intertrial interval of 10 s and then averaged.

MOVING STIMULATION ELECTRODE. To determine if activation of structures close to the parasubiculum might contribute to response components, in some experiments, the recording electrode was placed near layer II, and the location of the stimulating electrode was varied. Experiments were conducted after CSD recordings, and the electrode was moved in 200-μm steps between 2 mm ventral to 2 mm dorsal to target coordinates. Ten responses were recorded and averaged at each depth using an intertrial interval of 10 s.

HISTOLOGY. After electrophysiological recordings, cathodal current (100 μA) was passed through both electrodes to mark tip locations. Animals were perfused intracardially with 0.9% saline followed by 10% formalin. Brains were stored in 10% formalin and transferred to a 20% sucrose solution 1 day prior to sectioning. Sagittal sections (40 μm) were stained with formal-thionin and digital photographs of sections containing electrode tracks were taken. Multiple recordings were sometimes obtained from the surface of the entorhinal cortex in cases when the electrode was advanced too far (typically 50 μm), and traces were removed from current source density analysis on the basis of locations of electrolytic lesions. Measures of laminar thicknesses were determined using the software package Scion Image (Scion) following the conventions of Amaral and Witter (1995). Tissue obtained from animals with chronic electrodes (following text) was processed in the same manner.

CURRENT SOURCE DENSITY ANALYSIS. To localize the synaptic currents underlying field potentials, current source density (CSD) analysis was performed on depth profile recordings using computational methods reported previously (Chapman and Racine 1997a; Chapman et al. 1998a). In one-dimensional CSD analysis, membrane currents (I_m) are related to the second derivative of the extracellular voltage (V) measured as a function of cortical depth (z) by the equation

\[-I_m = \sigma_z \cdot \frac{d^2 V}{d z^2}\]

where \(\sigma_z\) reflects the conductivity of extracellular space. Field potentials are generated by inward and outward membrane currents, and the second derivative (i.e., changes in the voltage gradient) serves to localize the spatial origin of these currents. Variations in conductivity are typically assumed to have minimal effects on the results of CSD analysis, and the second derivative of the potential gradient (\(V^2\)) can therefore be used to estimate the CSD in arbitrary units (Mitzdorf 1985). This was implemented by differentiating a seventh-degree polynomial interpolated through data points. Because the second derivative is strongly affected by high-frequency noise, depth profiles were digitally smoothed prior to calculating the second derivative using a Blackman window that removed components with periods <200 μm.

Chronic recordings

SURGERY. Male Long-Evans hooded rats (300–350 g; n = 11) were anesthetized with sodium pentobarbital (65 mg/kg ip) and placed in a
stereotaxic apparatus with bregma and lambda leveled. Bipolar stimulating electrodes (tip separation of 0.6 mm) were lowered through burr holes into the right piriform cortex (P, 3.6 mm; L, 6.5 mm; V, 9.0 mm relative to bregma) and parasubiculum (P, 8.1 mm; L, 3.8 mm; V, 5.0 mm). A Teflon-coated stainless-steel recording electrode (125 μm exposed tip) was advanced into the superficial layers of the right medial entorhinal cortex (P, 8.5 mm and L, 4.8 mm relative to bregma, and 4.2 mm dorsal to the interaural line) at an angle 40° above horizontal. In three cases, the recording electrode was lowered vertically to the same coordinates. Vertical placements of stimulating electrodes were adjusted to minimize current thresholds, and the position of the recording electrode was adjusted to maximize response amplitudes. A stainless-steel jeweler’s screw in the contralateral frontal bone served as a reference electrode, and a second screw in the left parietal bone served as ground. Electrode leads were connected to gold-plated Amphenol pins and mounted in a plastic nine-pin connector. The entire assembly was embedded in dental cement and anchored to the skull with stainless-steel jeweler’s screws. Animals were housed individually and were tested after a ≥10 day recovery period during the lights-on phase of a 12-h light-dark schedule.

STIMULATION AND RECORDING. Animals were placed in a 40 × 40 × 60-cm Plexiglas chamber surrounded by a Faraday cage, and recordings were obtained after animals had habituated and were in a quiet resting state. Stability of responses was assessed using input/output tests conducted every 2 days over a 5-day period. During each input/output test, 10 responses to stimulation of either the parasubiculum or piriform cortex were recorded and averaged at each of 10 intensities (100–1,000 μA) using a 10-s intertrial interval. Peak response amplitudes were measured relative to the prestimulus baseline.

To determine if evoked responses are likely activated monosynaptically or polysynthetically, frequency of following tests were conducted in which short ~100-ms-duration trains of pulses at frequencies ranging from 60 to 125 Hz were delivered to the parasubiculum at an intensity that evoked single-pulse responses ~75% of maximal levels. Monosynaptic pathways typically follow stimulation frequencies near 100 Hz, whereas polysynaptic pathways tend to fail at frequencies <50 Hz due to variability in cell firing and summation of polysynaptic potentials (see Berry and Pentreath 1976). Five trains were delivered at each frequency for averaging, and there was a 20-s intertrial interval.

To determine if piriform cortex and parasubicullar inputs likely synapse onto overlapping populations of entorhinal cortex neurons, occlusion tests were used in which stimulation pulses delivered to both sites were timed to evoke simultaneous responses in the entorhinal cortex. With low-intensity pulses, simultaneous activation of either separate or overlapping populations of cells should result in responses that are equivalent to the summation of responses evoked by stimulating each input individually. In contrast, high-intensity pulses that cause much greater activation of the same population of neurons can result in an occlusion effect in which the response to combined stimulation is smaller than expected from summation (Chapman and Racine 1997a). During these tests, pulse intensities were set to evoke either maximal responses or responses that were ~50% of maximal, and responses evoked by single pulses were compared with responses evoked by combined stimulation. Due to differences in peak latencies of responses, the piriform cortex was stimulated 5.3–10 ms prior to the parasubiculum. Ten responses were recorded and averaged for each condition, and there was a 15-s intertrial interval.

Paired-pulse tests were used to assess the time courses of short-term facilitation and inhibition effects in the entorhinal cortex evoked by parasubicullar stimulation. In these tests, the effect of inhibitory and facilitatory mechanisms evoked by the first pulse were assessed by monitoring changes in the response to the second pulse. Pairs of stimulation pulses, separated by interpulse intervals ranging from 5 to 1,000 ms, were delivered to the parasubiculum using pulse intensities that evoked responses ~75% of maximal. Fifteen responses were recorded for averaging at each interpulse interval, and there was a 10-s intertrial interval.

Double-site paired-pulse tests were used to determine if stimulation of the parasubiculum can modulate responses in the entorhinal cortex evoked by piriform cortex stimulation. A conditioning pulse was delivered to the parasubiculum prior to the delivery of a test pulse to the piriform cortex at a variable delay interval (~1,000 ms). The conditioning pulse was set to evoke maximal responses, and the intensity of the test pulse was set to evoke responses that were ~75% of maximal. There was an intertrial interval of 10 s, and 15 responses were recorded for averaging at each interval. Because responses to test pulses at short intervals can be distorted by the response to the first pulse, at intervals <100 ms (in both single- and double-site tests), the amplitudes of responses were measured after subtracting the single-pulse evoked response from the waveform. Amplitudes of responses to test-pulses were expressed as a percentage of amplitudes of responses to single-pulses delivered to the piriform cortex.

RESULTS

Acute recordings

HISTOLOGY. Synaptic field potential responses were evoked in the entorhinal cortex when the stimulating electrode was positioned in or near the parasubiculum (Figs. 1A–3). Stimulation current may also have spread in some cases to activate low-threshold substrates in the caudal portion of the presubiculum, the corpus callosum, or deep layers of the entorhinal cortex. Recoding electrode trajectories were nearly perpendicular to the laminar plane, and the overall thickness of the medial entorhinal cortex at these sites ranged from 1,400 to 1,660 μm.

FIELD POTENTIAL DEPTH PROFILES. Stimulation of the parasubiculum evoked a similar pattern of field potentials in the entorhinal cortex in 9 of 12 animals (including the two animals in which data were recorded as the recording electrode was advanced toward the cortical surface). The main component of responses was a superficial negative deflection that peaked at depths between 250 and 400 μm below the surface (Fig. 1A, ●) and that reversed in polarity at depths of 500–700 μm. The negative component had a mean onset latency of 3.9 ± 0.8 (SE) ms, and a peak amplitude of 0.53 ± 0.16 mV at a latency of 7.5 ± 0.9 ms. The surface-negative component was preceded by one or two negative spike components with latencies near 2–4 ms (Fig. 1A, †). The spikes were maximal at depths of 50–650 μm and reversed polarity in both deep and superficial sites in seven animals. In four cases, a long-latency negative component, with onset and peak latencies of 10.8 ± 1.7 and 21.0 ± 1.2 ms, was also observed (Fig. 1A, ●). This component was relatively small (0.10 ± 0.03 mV), peaked at depths of 350–1,350 μm and showed no clear reversal. Paired-pulse stimulation caused a clear facilitation of the major surface-negative synaptic component in seven of nine animals (137.7 ± 14.0% of single-pulse levels for the group; t<sub>9</sub> = 2.39, P < 0.05) but did not enhance the spike or the small late negative component.

CSD ANALYSIS. Current source density analysis showed that the major surface-negative component was generated by a current sink in layer II (Fig. 1B), and in two cases, the sink also included an adjacent portion of layer III. The current sink peaked in layer II at depths of 300–450 μm below the cortical
surface at a mean latency of 7.1 ± 1.2 ms. Adjacent current sources were located in layers I and III (Fig. 1B), and the layer III source sometimes bordered on, or included, the lamina dissecans (3 of 7 cases). Paired-pulse stimulation caused a nonsignificant facilitation of the layer II current sink to 128.4 ± 19.3% of single-pulse levels.

The early spike components were associated with current sinks in layers II and III. In three cases, the sinks peaked at the layer II–III border, suggesting synchronous activation of layer II and III neurons (e.g., Fig. 1B), and the sinks were restricted to layer II in two cases, and to layer III in the remaining four cases. Corresponding current sources were located above and below the sinks at sites in layers I–II, and in layer III and the lamina dissecans.

The small, longer-latency deep-negative potential observed in four animals was associated with very long-lasting current sinks in layer III and/or V. All four rats displayed current sinks that peaked along the IV–V border at depths of 950–1,100 μm, and in three cases, a current sink was also observed in layer III (e.g., Fig. 1B).

In three additional animals, stimulation of sites outside of the parasubiculum (Fig. 2, A, open squares, and B, dashes lines) evoked field potentials that were not associated with a layer II sink (data not shown). In one animal, stimulation near the presubiculum evoked an initial spike associated with a layer III sink, and a subsequent surface-negative synaptic potential associated with a layer III current sink that peaked at a latency of 3.8 ms, 750 μm below the cortical surface. In two animals, stimulation of sites ventral to the parasubiculum near the entorhinal cortex evoked deep-negative field potentials associated with layer III current sinks at latencies of 2.8 and 3.7 ms.

MOVING STIMULATING ELECTRODE. To determine if stimulation of sites outside the parasubiculum may have contributed to the layer II sink, recordings in four animals were obtained while the position of the stimulating electrode was varied in 200-μm steps above and below target coordinates. The surface-negative component was activated most strongly when the stimulating electrode was in or near the parasubiculum (Fig. 3). Spike components were evoked by stimulation of sites within, and dorsal to, the parasubiculum, and an additional longer-latency spike (peak latency of ~9 ms) was observed in three animals as the electrode passed into the corpus callosum. The long-latency spike was not observed in one animal that had dorsal stimulation sites in visual cortex. In the example shown in Fig. 3, the largest responses were observed as the electrode entered the dorsal parasubiculum. Because fibers leaving the parasubiculum project ventrally to the entorhinal cortex (Caballero-Bleda and Witter 1993), the smaller responses observed at more ventral stimulation sites likely resulted in part from activation of sites ventral to the entorhinal cortex recording electrode.

Chronic recordings

INPUT/OUTPUT TESTS. Field potentials evoked by stimulation of the parasubiculum in freely behaving rats were similar to those in acute recordings and had a peak amplitude of 0.47 ± 0.06 mV at a latency of 7.4 ± 0.6 ms (Fig. 4A, n = 11). Field potentials evoked by piriform cortex stimulation were similar to those described previously (Chapman and Racine 1997a; Bouras and Chapman 2003) and contained a negative synaptic component with a peak amplitude of 0.36 ± 0.04 mV at a latency of 16.9 ± 0.5 ms (Fig. 4B). Current thresholds were 200–400 μA for the parasubiculum and 100–400 μA for the piriform cortex, and responses increased linearly with stimulation intensity.
activation of the entorhinal cortex with low-intensity pulses likely synapse onto the same pool of neurons. Simultaneous whether inputs from the parasubiculum and piriform cortex

**FREQUENCY OF FOLLOWING TESTS.** To assess whether synaptic responses in the entorhinal cortex are likely generated monosynaptically, frequency of following tests were conducted. Responses to individual pulses during short stimulation trains at frequencies of 60–125 Hz were always clear at 60 Hz but were less distinct as stimulation frequency was increased >90 Hz (n = 7). Responses clearly failed at 70 Hz in two cases and were maintained at frequencies between 80 and 125 Hz in the remaining animals (Fig. 5), consistent with a monosynaptic projection.

**OCCLUSION TESTS.** Occlusion tests were conducted to assess whether inputs from the parasubiculum and piriform cortex likely synapse onto the same pool of neurons. Simultaneous activation of the entorhinal cortex with low-intensity pulses produced responses that were no different from expected from

**PAIR PULSE TESTS.** To assess the time-course of inhibitory and facilitatory mechanisms in the entorhinal cortex evoked by parasubicular stimulation, pairs of stimulation pulses were delivered to the parasubiculum separated by interpulse intervals ranging from 5 to 1,000 ms (Fig. 7). Responses at the 5- and 10-ms intervals were significantly inhibited to 17.8 ± 10.7 and 41.7 ± 12.0% of single-pulse levels, respectively \([F(14,126) = 7.26, P < 0.0001; \text{N-K}, P < 0.01; n = 10]\). The maximal paired-pulse facilitation of 155.3 ± 21.5% was observed when the interpulse interval was 30 ms (N-K, \(P < 0.01\)).

Heterosynaptic inhibition and facilitation of piriform cortex responses was assessed by delivering single conditioning pulses to the parasubiculum followed by delivery of test-pulses to the piriform cortex at variable delay intervals (Fig. 8). Responses to piriform cortex stimulation were significantly inhibited at the 5-ms interval, to 67.9 ± 18.3% of single-pulse levels \([F(14,140) = 7.12, P < 0.0001; \text{N-K}, P < 0.0001; n = 11]\), and were facilitated at interpulse intervals of 20–150 ms (158.9 ± 17.2% at 20 ms). Activation of parasubicular inputs to the entorhinal cortex can therefore inhibit or facilitate subsequent synaptic responses to piriform cortex stimulation in a manner that depends on the timing of activation in the two pathways.

**DISCUSSION**

Field potential recording techniques have been used here to characterize parasubicular inputs to the entorhinal cortex in anesthetized and freely behaving rats and to determine how this pathway modulates entorhinal cortex responses to inputs from the piriform cortex. Consistent with the direct projection from layer I and II of the parasubiculum to layer II of the entorhinal cortex (Caballero-Bleda and Witter 1993; van Groen and Wyss 1990), stimulation of the parasubiculum resulted in a monosynaptic surface-negative potential that is generated by inward synaptic currents in layer II (Figs. 1, 3, and 5). Stimulation of the parasubiculum resulted in the time-dependent inhibition or facilitation of inputs to the entorhinal cortex from the piriform cortex (Fig. 8), indicating that the parasubiculum may play a role in modulating the responsiveness of layer II of the entorhinal cortex to extrahippocampal cortical inputs. Further, inputs to the parasubiculum from area CA1 and the subiculum and from amygdalar and thalamic nuclei (Köhler 1985; van Groen and Wyss 1990, 1992, 1995), provide routes through which ongoing activity in these structures can influence activity in entorhinal cortex projection neurons.

**CSD analysis**

Field potentials in the entorhinal cortex evoked by stimulation of the parasubiculum in anesthetized animals contained a major surface-negative component and one to two shorter-
latency population spike-like components. Results of CSD analysis indicated that the major negative synaptic component was generated by a current sink in layer II and that the early spikes were generated by current sinks in layers II and III. The synaptic component was most strongly activated when the stimulating electrode was located in or near the parasubiculum (Fig. 3), and the inward currents observed in layer II are consistent with activation of excitatory afferents from the parasubiculum (Caballero-Bleda and Witter 1993, 1994; Jones 1990). The latency of the synaptic component is also consistent with the 4.2-ms onset latency of intracellular EPSPs evoked by parasubiculum (PaS). Note the short-latency spike evoked at dorsal sites near the parasubiculum, and the long-latency spike evoked by stimulation near the corpus callosum (PrS, presubiculum; S, subiculum; MEC, medial entorhinal cortex; LEC, lateral entorhinal cortex; V2, secondary visual cortex).

FIG. 4. A and B: results of input/output tests after stimulation of the parasubiculum (A) and piriform cortex (B) in freely behaving animals. Representative field potentials in the entorhinal cortex are shown for the indicated pulse intensities (A1 and B1) and mean peak amplitudes of responses are shown as a function of pulse intensity (A2 and B2). Data indicate the means ± SE in this and subsequent figures.
stimulation of sites in the subicular complex (Finch et al. 1986). Therefore although we have not correlated the fEPSP recorded here with simultaneous unit recordings, both the known anatomy and previous intracellular recordings are consistent with its generation by excitation of layer II neurons. Parasubicular inputs to layer II are also known to terminate onto dendrites of layer III neurons, but the number of synaptic contacts is much less than for layer II neurons (Caballero-Bleda and Witter 1994). Synaptic activation of dendrites of layer V neurons that pass through layer II is also possible, but these connections have not been reported.

The initial short-latency spike most likely reflects synchronous antidromic activation of entorhinal cortex layer II and/or III neurons by direct stimulation of the perforant path and/or temporoammonic path. Antidromic spikes in layer II and III neurons have also been recorded intracellularly at similar latencies after stimulation of the subicular complex and hippocampal formation in vivo (Finch et al. 1986, 1988). Short-latency spikes that sometimes followed the initial spike likely reflect synchronized firing resulting from both synaptic inputs and voltage-dependent conductances activated by the initial spike.

FIG. 6. Results of occlusion tests in which stimulation of the PaS and piriform cortex (PIR) was timed to activate the entorhinal cortex simultaneously (PaS+PIR). A: averaged responses evoked by stimulating each site alone using low-intensity pulses are compared with the response observed following combined stimulation (A1, PaS+PIR, - - -), and the response expected from summing the individual responses (- - -). There was no significant difference between the amplitudes of observed (Obs) and expected (Exp) responses for low-intensity pulses (A2). B: combined delivery of high-intensity pulses resulted in responses that were smaller than expected on the basis of summation. Conventions are as in A, and ✉ highlights the response expected from summation. The difference between observed and expected response amplitudes was significant only for high-intensity pulses (B2; *, P < 0.01), suggesting that parasubicular and piriform cortex efferents terminate on overlapping populations of entorhinal cortex neurons.

FIG. 7. Paired-pulse stimulation of the PaS caused inhibition of responses at the 5- and 10-ms intervals, and facilitated responses at the 30-ms interpulse interval. A: representative averaged field potentials evoked by single pulses and by pairs of pulses at intervals of 10, 30, and 1,000 ms are shown. Note the absence of an observable response at the 10-ms interval (↑) and the large facilitation of the response at the 30-ms interval. - - - , the mean amplitude of responses to single pulses for comparison. B: mean amplitudes of responses to 2nd pulses are expressed as a percentage of amplitudes of responses evoked by the 1st pulse for each interpulse interval. *, significant differences from single-pulse levels (*, P < 0.01; **, P < 0.0001).

FIG. 8. Delivery of a strong conditioning pulse to the parasubiculum causes a reduction in the amplitude of responses evoked by piriform cortex stimulation 5 ms later but facilitates responses evoked by piriform cortex stimulation at longer delay intervals. A: averaged field potentials evoked by single pulses delivered to the piriform cortex and by double-site paired-pulse stimulation at intervals of 5, 40, and 1,000 ms are shown. - - - , the mean amplitude of responses evoked by single-pulse piriform cortex stimulation pulses. The size of the response evoked by piriform cortex stimulation was reduced (↑) when the parasubiculum was stimulated 5 ms earlier but was facilitated by stimulation of the parasubiculum at the 40-ms interpulse interval. B: mean amplitudes of responses evoked by piriform cortex stimulation at various intervals after stimulation of the parasubiculum are expressed as a percentage of the amplitudes of responses evoked by single-pulses delivered to the piriform cortex. Responses were inhibited at the 5-ms interpulse interval and were facilitated at intervals of 20–100 ms (*, P < 0.05; **, P < 0.0001).
The initial spike appears to result from antidromic activation of entorhinal cortex neurons, but a resulting activation of recurrent collaterals between layer II neurons is unlikely to have contributed substantially to the synaptic response. Axon collaterals of layer II neurons can branch extensively (Dolorfo and Amaral 1998; Klink and Alonso 1997), but recurrent connections between principal neurons in layer II appear to be rather sparse. Paired intracellular recordings have shown quite high probabilities of recurrent connections between layer III and layer V neurons in the rat but have failed to find connections between layer II neurons (Dhillon and Jones 2000; but see Biella et al. 2002 in the guinea pig), and it has been suggested that many axonal arborizations of layer II neurons target inhibitory cells (Dhillon and Jones 2000). Although the antidromic spike often involved substrates in layer III (5 of 7 cases) and recurrent collaterals are relatively common between layer III neurons (Dhillon and Jones 2000), the synaptic component was associated with activation that included a portion of layer III in only one case. This suggests that the synaptic component is due primarily to monosynaptic activation of layer II neurons by parasubicular inputs rather than being driven by activation of layer II or III axonal collaterals or by monosynaptic inputs to layer III.

In four cases, weak deep-negative responses were associated with sinks in layers V and/or III, but the circuitry that mediates these long-latency, low-amplitude responses is not clear (e.g., Fig. 1). The long latencies and durations of these responses makes it unlikely that they were generated by direct activation of projections from the presubiculum, subiculum or CA1 region, but these pathways may have been driven polysynaptically by activation of the dentate gyrus, CA3, and/or subiculum (Witter et al. 1989).

Interactions between parasubicular and piriform cortex inputs

Field potentials in awake, freely behaving rats were similar to those observed in anesthetized animals and were used to assess interactions between inputs to the entorhinal cortex from the parasubiculum and piriform cortex. Stimulation of either input evoked field potential responses at the same recording site (Fig. 4), and results of occlusion tests were consistent with the termination of both input pathways on a common population of layer II neurons (Fig. 6). The occlusion of responses observed during simultaneous activation of entorhinal targets (Chapman and Racine 1997a) might be explained partly by a nonlinear increase in activation of feedforward inhibition by high-intensity pulses, but field potentials in the medial entorhinal cortex after piriform cortex stimulation are known to result from activation in layer II (Chapman and Racine 1997a), and peak latencies of inhibitory responses evoked by various inputs to the entorhinal cortex tend to be 5–10 ms longer than for EPSPs (Finch et al. 1986, 1988).

Paired-pulse tests assessed the time course of inhibitory and facilitatory mechanisms evoked by parasubicular stimulation. At the shortest interpulse intervals, stimulation of the parasubiculum inhibited responses to both parasubicular and piriform cortex inputs (Figs. 7 and 8). The inhibition of parasubicular responses occurred at the 5- and 10-ms interpulse intervals, and inhibition is observed at similar intervals after stimulation of the piriform cortex (Bouras and Chapman 2003; Chapman and Racine 1997a) and amygdala (Colino and Fernandez de Molina 1986). Stimulation of the subicular complex in vivo, which included sites within the parasubiculum (Fig. 1, in Finch et al. 1986), also evokes inhibitory postsynaptic potentials (IPSPs) in layer II neurons with a latency of ~10 ms (Finch et al. 1986, 1988). Stimulation of various sites in the hippocampal formation results in IPSPs in layer II cells with latencies that are strongly correlated with the latencies of EPSPs rather than with antidromic responses, suggesting that inhibitory responses are generated primarily by feedforward rather than by feedback inhibition (Finch et al. 1988). Stimulation of the parasubiculum in vitro activates inhibitory interneurons in layer II of the entorhinal cortex (Jones and Buhl 1993) and results in prominent IPSPs in layer II principal cells (Jones 1990), suggesting that the inhibition observed here may be mediated primarily by feedforward inhibition.

Feedforward inhibition also accounts for the heterosynaptic paired-pulse inhibition of responses to piriform cortex stimulation at the 5-ms interval (Finch et al. 1988; Jones and Buhl 1993). The presence of inhibition at only the 5-ms interval for piriform inputs, rather than at 5 and 10 ms for parasubicular inputs, is predictable based on the longer peak latency of piriform cortex evoked responses; in both single- and double-site paired-pulses tests, the inhibition was observed when test-responses occurred ~10 ms after the conditioning pulse. In addition to synaptic inhibition, the heterosynaptic depression might also be explained in part by reductions in the driving force on EPSPs induced by prior activation of parasubicular inputs.

At longer interpulse intervals, there was a facilitation of responses to both parasubicular and piriform cortex stimulation. Reasons for the shorter time course of facilitation of parasubicular responses versus piriform cortex responses are not clear, but the heterosynaptic facilitation effect is similar to that observed in piriform cortex inputs following stimulation of the medial septum (Chapman and Racine 1997a), and a prominent facilitation at intervals of 30 and 40 ms is also observed after paired-pulse stimulation of the piriform cortex (Bouras and Chapman 2003; Chapman and Racine 1997a). Homosynaptic paired-pulse facilitation is often attributed to enhanced transmitter release due to residual presynaptic calcium (Zucker 1989), but this cannot explain heterosynaptic facilitation effects, and a number of additional mechanisms likely contribute. Responses evoked by piriform cortex (Alonso et al. 1990; Kourrich and Chapman 2003) and parasubicular inputs (Jones 1990) contain NMDA receptor-mediated components that could be enhanced by depolarization-induced weakening of the Mg2+ -block on the NMDA receptor channel. Layer IIstellate neurons could be depolarized in part by activation of the persistent sodium current (Magistretti et al. 1999), but a rebound depolarization mediated by the hyperpolarization-activated nonspecific cationic current I\(i_p\) (Alonso and Klink 1993; Dickson et al. 2000b,c) is more likely to contribute at longer intervals after the decay of inhibition. This current is a major contributor to paired-pulse facilitation in the sensorimotor cortex at long interpulse intervals (Castro-Alamancos and Connors 1996; Werk and Chapman 2003). In addition, the heterosynaptic facilitation could have resulted from enhanced glutamate release mediated by activation of presynaptic NMDA receptors by glutamate spillover from parasubicular terminals. Presynaptic NMDA receptors are known to contrib-
ute to homosynaptic facilitation effects in layer II neurons (Berretta and Jones 1996; Woodhall et al. 2001).

Reduced inhibitory synaptic transmission is also likely to have contributed to the facilitation effects observed here. Inhibition in the entorhinal cortex is reduced during repetitive stimulation (Jones 1993–1995), and paired-pulse facilitation in the CA1 is mediated in part by reduced inhibition during the response to the second pulse (Davies and Collingridge 1996; Leung and Fu 1994). Paired-pulse facilitation in the neocortex also results partly from activation of presynaptic autoreceptors that reduce GABA release during the second response (Deisz and Prince 1989; Metherate and Ashe 1994).

Together, the effects of parasubicular stimulation observed here are consistent with the activation of local inhibitory interneurons that inhibit layer II principal cells (Finch et al. 1988; Funahashi and Stewart 1998; Jones 1994, 1995; Jones and Buhl 1993), and the subsequent facilitation of responses evoked a short time later. The activation of entorhinal cortex projection neurons is therefore likely to be affected by the relative timing of inputs from the parasubiculum and other cortical regions.

Functional significance

Excitatory inputs from the parasubiculum to the entorhinal cortex provide a pathway through which part of the "output" of the hippocampal formation can re-enter the hippocampal circuit. Patterns of activity among parasubicular neurons can be shaped by inputs from the CA1 region and subiculum, and this provides a mechanism through which ongoing hippocampal processing can influence which entorhinal cortex projection neurons are most active and most responsive to sensory inputs. Similarly, the parasubiculum may also affect activation in the temporoparietoammonic pathway via synapses onto layer III neurons (Caballero-Bleda and Witter 1994) and in the dentate gyrus through its smaller projection to the molecular layer (Köhler 1985; Witter et al. 1988).

The parasubiculum is more likely to affect activity in the entorhinal cortex during theta- and gamma-frequency activity than during hippocampal CA1 sharp waves. Sharp waves are associated with increased firing in deep layers of the subiculum, presubiculum, parasubiculum, and entorhinal cortex (Chrobak and Buzsáki 1994), but the parasubiculum projection to the entorhinal cortex arises from layer II and III neurons (Funahashi and Stewart 1997; van Groen and Wyss 1990), and sharp-waves are not associated with increased firing in the superficial layers of the parasubiculum or entorhinal cortex (Chrobak and Buzsáki 1994). The parasubiculum receives a prominent input from the medial septum (Alonso and Köhler 1984; Gaykema et al. 1990), and Taube (1995) has observed that a substantial proportion of parasubicular cells (~41%) fire in relation to theta and often fire in rhythmic bursts. Gamma-frequency activity is generated in layer II of the entorhinal cortex by synchronous synaptic inputs during the negative phase of the theta rhythm (Chrobak and Buzsáki 1998; Dickson et al. 2000a), and maximal paired-pulse facilitation effects were observed at an interval similar to the period of the gamma rhythm (Fig. 7). Theta- and gamma-frequency activities in the entorhinal cortex may therefore be accompanied by inputs from the parasubiculum that may enhance the responsiveness of layer II neurons to cortical sensory inputs. This may affect the processing of olfactory information and could also contribute heterosynaptically to lasting changes in synaptic strength (Chapman and Racine 1997b; Kourrich and Chapman 2003).

GRANTS

This research was funded by grants to C. A. Chapman from the Natural Sciences and Engineering Research Council of Canada, the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche, and the Canadian Foundation for Innovation.

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J Neurophysiol • VOL 92 • AUGUST 2004 • www.jn.org


