

Associative Interactions Within the Superficial Layers of the Entorhinal Cortex of the Guinea Pig

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Received 10 January 2002; accepted in final form 21 May 2002

Biella, Gerardo, Laura Uva, Ulrich G. Hofmann, and Marco de Curtis. Associative interactions within the superficial layers of the entorhinal cortex of the guinea pig. *J Neurophysiol* 88: 1159–1165, 2002; 10.1152/jn.00022.2002. Associative fiber systems in the entorhinal cortex (EC) have been extensively studied in different mammals with tracing techniques. The largest contingent of intra-EC cortico-cortical fibers runs in the superficial layers and is distributed predominantly within longitudinal cortical bands. We studied the patterns of intrinsic EC connectivity in the in vitro isolated guinea pig brain preparation by performing current-source density analysis of field potential laminar profiles recorded with multi-channel silicon probes. The response pattern evoked by stimulation of the lateral olfactory tract was utilized to identify the lateral (l-EC) and medial (m-EC) entorhinal cortex. Stimulation of the deep layers did not evoke consistent responses. Local stimulation of the superficial layers in different portions of the EC induced an early, possibly direct response restricted to layer II–III in the close proximity to the stimulating electrode, followed by a late potential in the superficial layer I, that propagated at distance with a progressively increasing latency. The monosynaptic nature of the delayed response was verified by applying a pairing test. The results demonstrated that stimulation in the rostral-medial part of the EC generated activity restricted to the rostral pole of the l-EC, stimulation of the m-EC induced an associative activation that propagated rostrocaudally within the m-EC, stimulation of the caudal pole of the m-EC induced an additional response directed laterally, and stimulation of the lateral band of the EC determined a prominent longitudinal propagation of neuronal activity, but also induced associative potentials that propagated medially. The results are in partial agreement with the general picture derived from the anatomical studies performed in different species. Even though the largest associative interactions between superficial layers are restricted within either the m-EC or the l-EC, both rostral and caudal stimuli in the EC region close to the rhinal sulcus induced activity that propagated across the border between l- and m-EC.

INTRODUCTION

The entorhinal cortex (EC) is one of the regions of the parahippocampal area that conveys cortical inputs into and receives a feedback projection from the hippocampus (Lopes da Silva et al. 1990; Witter et al. 1989). The complex pattern of connectivity between the EC and the hippocampus has been extensively characterized in different animal species. On the basis of the projection pattern of layer II neurons to the dendrites of dentate gyrus granule cells (Brodman 1909;

Hjorth-Simonsen and Jeune 1972; Shipley 1975; Steward 1976; Steward and Scoville 1976; Swanson and Kohler 1986) and of layer III neurons to the CA1-subicular region (Witter 1993), the EC has been divided in two major subfields, medial (m-EC) and lateral (l-EC). The intrinsic EC connectivity in the rat is characterized by longitudinal fibers confined to the lateral band of cortex close to the rhinal sulcus that connect caudal and rostral portions of the EC and by transversal fibers at different caudal-dorsal level within the m-EC (Dolorfo and Amaral 1998). Intrinsic associative fibers within the EC are located predominantly in superficial layers and, less extensively, in the deep layers (Dolorfo and Amaral 1998; Kohler 1986, 1988). The anatomical data therefore devise a complex pattern of intrinsic interactions within and across the border between m- and l-EC.

We verified with electrophysiological techniques the pattern of intrinsic associative connections in the EC of the in vitro isolated brain of the guinea-pig (de Curtis et al. 1991, 1998; Llinás et al. 1981; Muhlethaler et al. 1993), a preparation that allows for a facilitated access to the entorhinal region under direct visual control. We recently demonstrated that the border between m- and l-EC in the guinea pig can be inferred by the activation pattern induced by direct olfactory input, which is largely confined to the l-EC (Biella and de Curtis 2000) and by the ability to generate fast oscillatory activity on muscarinic activation, a peculiar property of the m-EC (van der Linden et al. 1999). The identification of the borders between m- and l-EC was recently morphologically verified in the guinea pig by performing a cytoarchitectonic study of the region (Uva et al. 2001) based on the criteria utilized for the rat (Insausti et al. 1997).

METHODS

Brains of young adult guinea-pigs (150–200 g) were dissected out according to the standard procedure (de Curtis et al. 1991, 1998) after anesthesia with Farmotal (20 mg/kg ip; Pharmacia-Upjohn, Milano, Italy). A solution containing (in mM) 126 NaCl, 3 KCl, 1.2 KH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂, 26 NaHCO₃, and 15 glucose mM and 3% dextran MW 70.000, oxygenated with a 95% O₂-5% CO₂ gas mixture (pH 7.3) was arterially perfused in vitro at 5.5 ml/min. Experiments were performed at 32°C. Bipolar stimulation was delivered with tungsten electrodes arrays formed by two electrode pairs at 200 μm

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vertically separated by 500–1,000 μm (FHC, Bowdoinham, ME) positioned at different depths in EC. Current stimuli of 50–150 μA and 200–500 μs were applied. Extracellular laminar depth profiles were performed with silicon probes (16-recording sites separated by 50 μm on a single vertical shaft; kindly provided by Jammie Hetke of the Center of Neural Communication and Technology of the Michigan University, Ann Arbor, MI). The position of the electrodes was easily and rapidly modified during the experiment under direct visual control via a stereoscopic microscope. Signals were amplified with a 16-channel extracellular amplifier (Biomedical Engineering, Thornwood, NY), were digitized via an AT-MIO-64E3 National board (National Instruments, Milano, Italy) and were stored on a tape recorder (Biologic Instruments, Claix, France). Off-line analysis was performed by using a software (CLAMPVIEW) developed in our department by G. Biella in collaboration with the Italian branch of National Instruments. A different acquisition and analysis system (Folkers and Hofmann 2001) was also utilized to perform on-line acquisition of laminar profiles. Current-source density analysis (CSD) was implemented with 50 μm steps on 200 μm depth intervals according to the standard procedure previously described (Biella and de Curtis 1995, 2000; Ketchum and Haberly 1993).

Electrolytic lesions performed at the end of the experiments were utilized to mark the position of the electrodes (see METHODS in Biella and de Curtis 2000). After fixation in 4% paraformaldehyde, 75- μm sections were cut by vibratome and were processed for thionin staining. The location of the stimulating and recording probes in the EC was identified by marking their position on a tri-dimensional reconstruction of the guinea pig EC (Uva et al. 2001). The location of the electrodes was reproduced on a photograph of the brain obtained during the electrophysiological experiment with a camera connected to the stereoscopic microscope.

RESULTS

Recordings were performed from either one or both hemispheres of 18 guinea pig brains. Stimuli were delivered at different rostrocaudal levels in the EC (Fig. 1). The position of the recording and stimulating electrodes were reproduced in different experiments by using a large-size arterial branch of the limbic artery as surface reference point. Recordings were obtained with 16-channel silicon probes at 154 EC

sites (Fig. 1B) located either in the medial or lateral cortical bands of both the m-EC and the l-EC as defined by the response to lateral olfactory tract (LOT) stimulation (see Biella and de Curtis 2000). As previously demonstrated, l-EC responses were characterized by a large wave component at 20–25 ms that represents the direct propagation of the olfactory input (Fig. 1C, top), whereas the m-EC was characterized by a predominant delayed component at 50–60 ms (Fig. 1C, bottom) that represented a polysynaptic potential mediated through the activation of the hippocampus (Biella and de Curtis 2000).

Stimuli were delivered both in superficial and deep layers at each EC stimulation site. Superficial layer stimuli induced highly reproducible responses, whereas deep layer stimuli determined either no responses at a distance longer than 1 mm from the stimulating electrode. Depending on the intensity of deep layer stimulation, highly variable responses were observed in the EC close to the stimulating electrode. Because we could not evoke reproducible potentials in response to deep layer stimulation, we decided to restrict the study of long-range activity propagation to responses to superficial layer stimulation.

Regardless of the position of the recording and stimulating electrodes in the EC, we identified two typical, quite stereotyped patterns that were further analyzed with CSD analysis of laminar profile (Fig. 2, left). In almost all the experiments, the response pattern distinctively recorded within 1.2 mm from the stimulating electrode was characterized by a biphasic response (Fig. 2, top left). The early potential (positive at the surface and negative at depth) showed an average peak latency shorter than 10 ms and correlated to a current sink that extended between 200 and 600 μm in layers II and III shown in the CSD contour plot. As illustrated in the example in Fig. 2, in the large majority of the experiments such an early current was divided in two separate sinks centered around 200- and 500- μm depths. The histological control of the silicon probe track confirmed

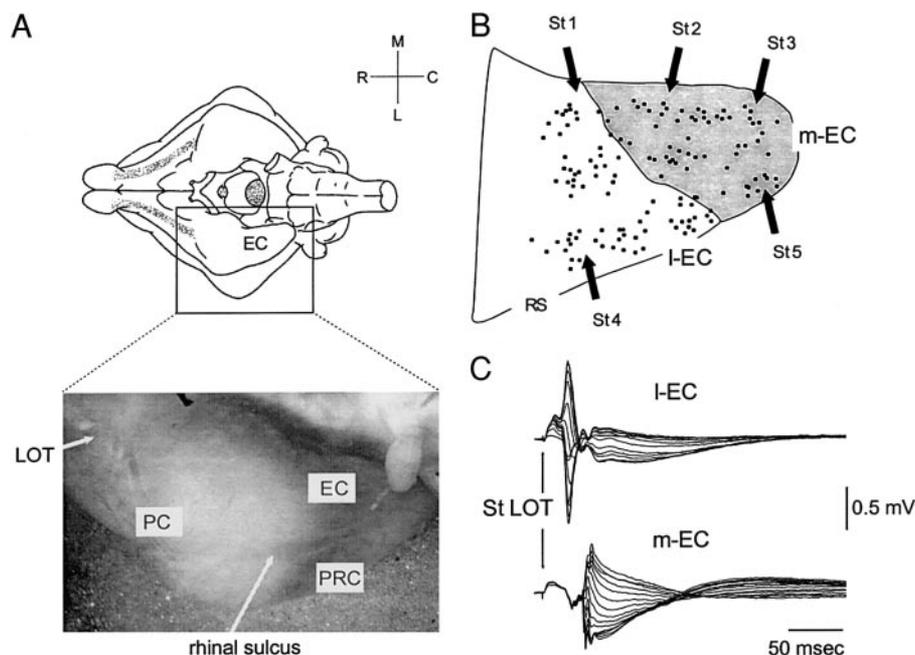


FIG. 1. A: drawing of the ventral view of a guinea pig brain and photograph of the entorhinal region taken after the electrophysiological recording. PC, piriform cortex; EC, entorhinal cortex; PRC, perirhinal cortex; LOT, lateral olfactory tract. B: scheme of the positions of the recording 16-channel silicon probes (●) and the stimulating electrodes (→) in the entorhinal area (summary of 18 experiments). The drawing of the EC was enlarged from the ventral view of the isolated guinea pig brain in A. The borders of the EC were outlined from microphotographs of the EC obtained during the experiment, such as the 1 illustrated in A. The medial EC (m-EC), as defined by the pattern to LOT response observed at each recording site, is identified by the gray shading. C: typical responses of the lateral EC (l-EC, top) and the m-EC (bottom) to stimulation of the LOT

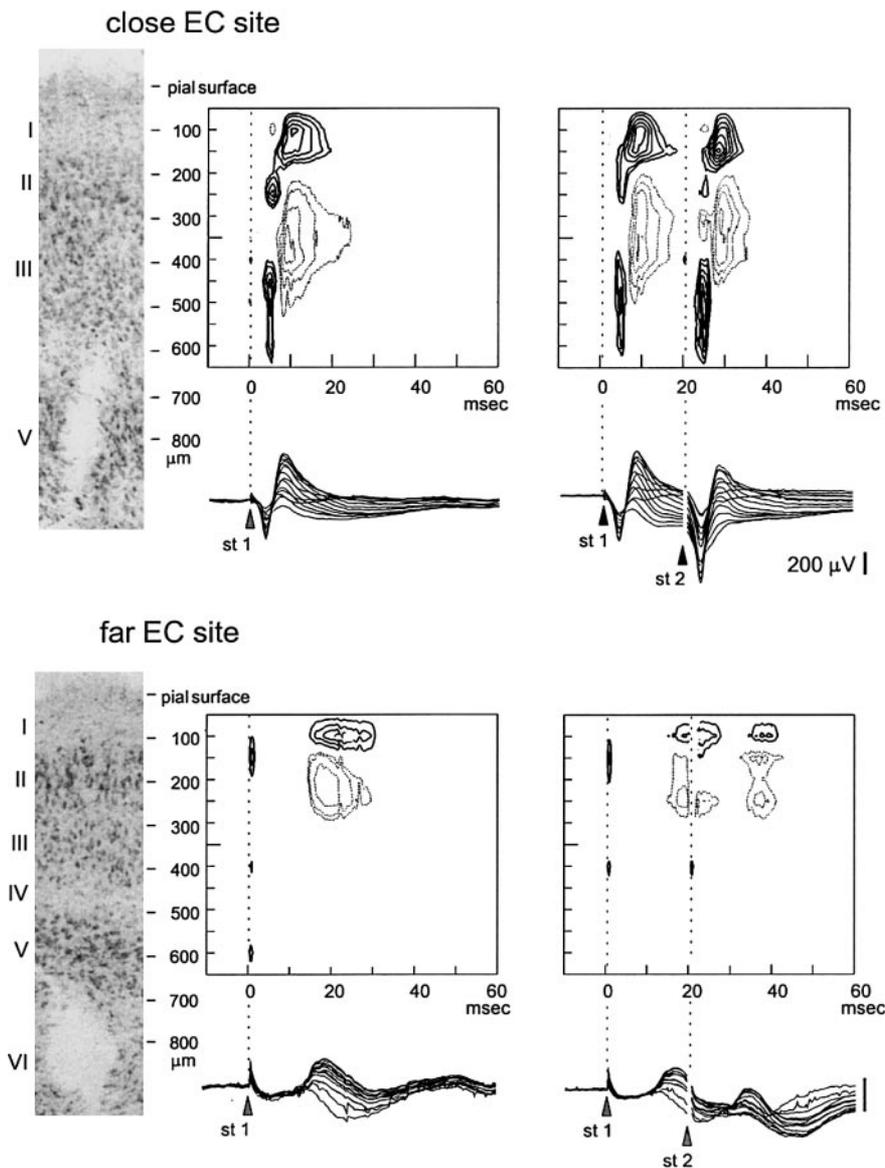


FIG. 2. Response patterns evoked by local EC stimulation. The 2 recording silicon probes were positioned close to and far from the stimulating electrode positioned caudal in the I-EC band close to the rhinal sulcus (site 5 in Fig. 3). *Left*: the thionin-stained coronal section illustrate the histological control of the lesion performed at the tip of the silicon probe (see METHODS). *Top left*: a typical biphasic response recorded in the EC close to the local stimulation. current-source density (CSD) analysis of the laminar profiles is illustrated in the contour plot. Sinks and sources are identified by — and ···, respectively. The depth and time distribution of sinks and source that generate the 2 components of the response are shown. The early current is formed by 2 separate sinks that correspond to layer II and layer III (identified on the histological control sections). A typical “pure” late associative responses generate far from the stimulating electrode is shown in the *bottom left panel*. *Right*: pairing stimuli demonstrate that the late response is monosynaptic. The superficial late sink is preserved in the second response to a paired stimulation with 20-ms interval. Note that the 1st associative response is superimposed to the artifact of the 2nd paired stimulus (blanked in the CSD contour plot). Isocurrent lines at 10 mV/mm². The time calibration of the field traces is the same as for the CSD plots.

the location of the two sinks in layer II and in layer III, respectively (see microphotographs). The early event was followed by a delayed response at 10–20 ms that correlated with a sink in the superficial molecular layer coupled with a current source in layer II–III.

At distance from the stimulation electrode, an isolated response with a longer latency was observed (Fig. 2, *bottom left*). Such a potential was associated with a superficial sink in layer I, which was coupled with a source in layer II. Paired-pulse stimulation at 10- to 30-ms inter-stimulus interval showed that both at sites close and far from the stimulating electrode the sink associated with the late potential was not abolished in the second, conditioned response (Fig. 2, *right*). In addition to the preservation of the late layer I sink, the paired response at a site close to the stimulation electrode showed a larger early potential/sink (Fig. 2, *top right*). These results strongly suggest that the superficial late event is mediated by an associative monosynaptic potential generated by the output of layer II–III neurons directly activated in the tissue around the stimulating electrode (see DISCUSSION).

The delay of the late synaptic response increased with the distance from the EC stimulation site. Figure 3 summarizes the pattern of propagation of both the direct and the late synaptic responses obtained from recordings performed at 154 sites in which EC stimulation was delivered at different sites (1–5). With a few exceptions, the responses characterized by the biphasic activation pattern were restricted to the EC portion in close proximity (approximately 1 mm) to the stimulating electrode (marked by the white-dot-in-black-circle symbol); the increase in delay of the associative synaptic response is illustrated by different gray shadings (see legend in Fig. 3). The pattern of tangential propagation of the associative synaptic potentials differed substantially for the different stimulation sites. Stimulation of the rostral part of the medial portion of the I-EC (site 1) induced a propagation restricted to the medial and rostral portion of the I-EC. Stimulation at an intermediate longitudinal position in the medial band of the m-EC (site 2) induced a longitudinal propagation directed both rostrally and caudally within the m-EC band. Little propagation directed

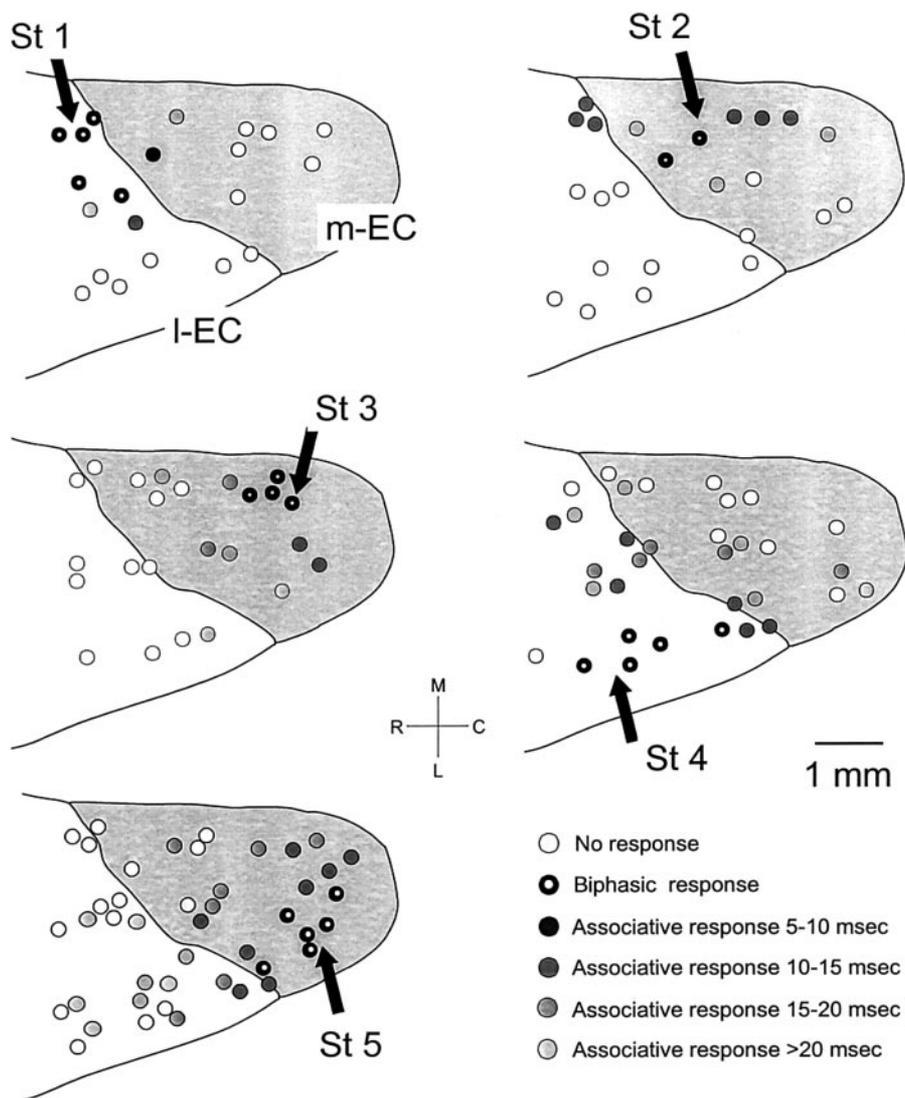


FIG. 3. Distribution of the synaptic responses to different local EC stimulation sites (1–5) in the superficial layers. The responses characterized by a biphasic potential are marked by the symbol characterized by a white dot on a black circle. The associative responses with peak amplitude values of gradually increasing delays are represented by circles of decreasing gray intensity. As for Fig. 1, the m-EC is shaded in light gray.

laterally was observed following stimuli at sites 1 and 2. Stimulation in a medial and caudal m-EC position (site 3) induced a propagation in the rostral and lateral direction limited to the caudal part of the m-EC. The associative activity induced by stimulation of the rostral part of the l-EC lateral band (site 4) propagated caudally and medially, across the border between m-EC and l-EC. Finally, caudal-lateral stimulation at site 5 determined a diffuse propagation within the m-EC, and a rostral projection at distance along the lateral band, to the l-EC.

The delay of the late responses increased with distance from the region directly activated by the local stimulation, as shown in the representative experiment illustrated in Fig. 4A. The peak latencies of the early component (●) and late component (▲) of the responses evoked by the local stimulus is illustrated in the graph in Fig. 4B. The plot includes biphasic responses (such as *a*) and pure monophasic potentials (such as *b* and *c*). The distribution of the delays was homogeneous in different directions of propagation for the different stimulation sites 1–5. The general direction pattern of associative projections derived from the experiments described in the preceding text is summarized in the scheme in Fig. 5.

DISCUSSION

The present study describes the pattern of activity propagation along the intrinsic associative fibers that run in the superficial plexiform layer of the EC. The study has been restricted to the activity generated within the superficial layers because no activity was observed in deep layers following superficial EC stimulation, probably because of the spatial and temporal dispersion of the associative connections in deep EC layers that prevents the identification of a field potential reversal or a current sink with CSD analysis (Mitzdorf 1985). In addition, we excluded from the study the analysis of the associative potentials evoked by deep layers stimulation because a highly variable response pattern was obtained in the EC close to the stimulating electrode following stimulation of deep layers and no clear responses were observed with CSD analysis of laminar profiles at cortical sites remote from the deep stimulation site.

Local cortical stimulation induced a spatially restricted neuronal discharge in layers II and III in close proximity (less than 1.2 mm) to the stimulating electrode, represented by the early CSD sinks located at 200- to 600- μ m depth. The identification of such depth values with layers II and III was accomplished by reconstructing the silicon probe position on a detailed cytoar-

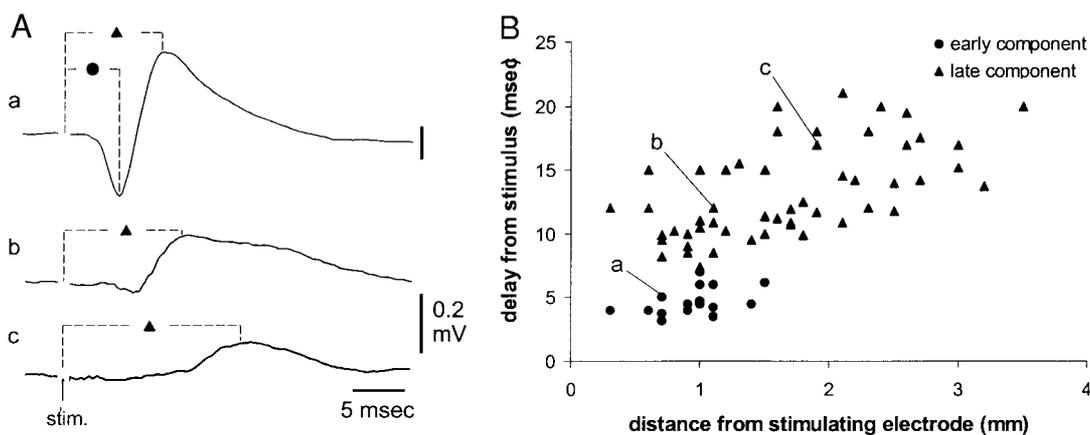


FIG. 4. Distribution of peak latencies of the early (●) and late (▲) components of the evoked response. The delay of the late component increased with the distance of the recording site from the stimulation electrode. A: recordings obtained at 3 sites 0.8 (a), 1.1 (b), and 1.9 mm (c) from the stimulating electrode positioned at site 5. The delays calculated on the peak amplitudes of the early and late components recorded in 11 experiments in which stimuli were applied at site 1 ($n = 3$), site 2 ($n = 2$), site 3 ($n = 2$), site 4 ($n = 4$), and site 5 ($n = 6$) are plotted in B. The latencies from the stimulus of the early and the late peak amplitude responses are plotted against the distance from the stimulation site. The values of the field potential components illustrated in A are indicated in the graph.

chitectonic map of the guinea pig EC (Uva et al. 2001). Such a response could be mediated by the direct activation of the neurons close to the stimulating electrode and/or by the monosynaptic activation of layer II–III cells. As expected from a response due to either the direct activation or the antidromic invasion of neuron somata in layers II–III, the early potential/sink showed a very short delay from the stimulus artifact (less than 5 ms) and was not abolished by high-frequency stimulation at and above 50 Hz, as demonstrated during the pairing tests performed with a 10- to 30-ms inter-stimulus intervals. In the lateral band of the l-EC, a possible direct activation of the olfactory input to the propagation pattern observed should be also taken in account because olfactory fibers that arise from both the olfactory bulb (Biella and de Curtis 1995, 2000; Boeijinga and Van Groen 1984; Haberly and Price 1977; Kosel et al. 1981; Krettek and Price 1977; Liu and Bilkey 1997;

Luskin and Price 1982; Schwerdtfeger et al. 1990; Van Groen et al. 1987; Wilson and Steward 1978; Wouterlood and Nedderlof 1983) and the piriform cortex (Boeijinga and Van Groen 1984; Chapman and Racine 1997a,b; Krettek and Price 1977; Luskin and Price 1983; Van Groen et al. 1987) are known to run in the superficial layers of the l-EC but not the m-EC. Even though, in principle, a monosynaptic excitatory response evoked by the activation of the olfactory input fibers could contribute to both the early response close to stimulation site and the late potential at remote sites, the results of the pairing test do not support this conclusion.

The observed results suggest that the late superficial synaptic responses (late CSD sinks) are sustained by the activation of cortico-cortical associative fibers that originate from the discharge of layer II–III cells directly activated by the local stimulus. These late potentials should be mediated through monosynaptic associative responses because their delay from the direct potential are compatible with a single synapse. Moreover, a polysynaptic origin of the late associative response/sink is excluded by the demonstration that it is preserved in the conditioned response during a pairing test (Biella et al. 1996).

The anatomical connectivity within the EC has been studied in detail in the rat (Dolorfo and Amaral 1998; Kohler 1986, 1988; Kosel et al. 1982; Swanson and Köhler 1986), in the cat (Room and Groenewegen 1986; Witter et al. 1986) and in the monkey (Kosel et al. 1982; Suzuki 1996). Because no anatomical data are available in the guinea pig, the present physiological finding will be discussed with reference with the data described in the rat. Our results demonstrate that stimulation in the medial-rostral EC (site 1) generates activity that remains localized in the mediorostral pole of the EC, stimulation of the medial band of m-EC (site 2) induced an associative propagation directed longitudinally, stimulation of the caudal part of the m-EC (site 3) induces a short range propagation in the lateral and rostral directions within the m-EC, and stimulation of the lateral band (sites 4 and 5) induces a prominent longitudinal propagation of activity across the m-EC/l-EC border. Unlike suggested by the anatomical studies, the stimulation

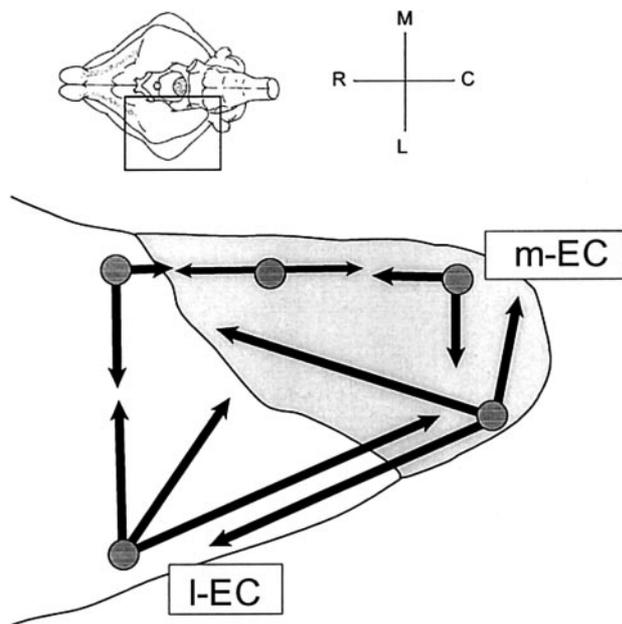


FIG. 5. Summary of the propagation direction of the associative potentials in the superficial layers of the EC.

within the m-EC band (sites 2 and 3) induces a longitudinal propagation of activity and the stimulation of the caudal pole of the m-EC in a lateral position (site 5) induces a diffuse propagation of the activity at distance in the rostral and medial directions. These discrepancies may be due to species differences. Indeed, even though preliminary cytoarchitectonic studies suggest that the general organization of the EC is similar between rat and guinea pig (Uva et al. 2001), the relative dimension of l- and m-EC and the subfields that compose these two regions, as well as the topographic organization of the projection of superficial layers to the hippocampus, may be different in the two species. A region of particular interest at this regard, for which an unequivocal attribution to either the m-EC or the l-EC has not defined yet, is the subfield denominated F3 that represents an extended portion of the guinea pig EC (Insausti et al. 1997; Uva et al. 2001). A specific ad hoc study will be necessary to clarify this issue.

The present findings demonstrate that both rostral and caudal stimuli in the EC region close to the rhinal sulcus induced activity propagation across the border between l- and m-EC. As for the rat (Insausti et al. 1997), the guinea pig EC can be subdivided in subfields that belong to either the MEA or the LEA, i.e., the medial and lateral EC regions that project to the inner and outer portion of dentate gyrus granule cells, respectively (Steward 1976; Steward and Scoville 1976). The results obtained in a recent collaborative study (Uva et al. 2001) strongly suggest that the identification of the border between the LEA and MEA coincide with the delimitation m- and l-EC defined on the basis of the electrophysiological response to LOT stimulation (Biella and de Curtis 2000). According to this pattern, l-EC neurons do not induce a prominent projection to the m-EC. This conclusion derived from the demonstration that a large-amplitude response in the m-EC could be induced exclusively when the hippocampus was activated. Unlike previously suggested, we recently observed that small amplitude responses could be recorded in the m-EC after LOT-induced l-EC activation, mostly in an intermediate band of the EC located between m- and l-EC, that probably coincides with the above mentioned subfield identified as F3 according to the classification of Insausti (Insausti et al. 1997). Such responses were not large enough to generate reproducible sinks during CSD analysis (not shown). Therefore even though olfactory-induced m-EC activation through the hippocampus is large and easy to detect, the existence of a direct intrinsic propagation of neuronal activity within the EC cannot be excluded and, indeed, is strongly suggested by the present study and by the anatomical study by Dolorfo and Amaral (1998). Such a longitudinal propagation of excitation, directed parallel to the rhinal sulcus, is similar to the propagation pattern of neuronal activity observed in the adjacent perirhinal cortex (Biella et al. 2001; Martina et al. 2001).

The results confirm that different portions of the EC are strongly interconnected by an associative system of fibers that likely sustain the complex integrative function performed by this region before neuronal activity is propagated to the hippocampus.

Multi-channel silicon probes were kindly provided by the University of Michigan Center for Neural Communication Technology, sponsored by Division of Research Resources Grant P41-RR09754.

The study was sponsored by the European Community grant Development

of a Versatile System for Advanced Neuronal Recordings with Multisite Microelectrodes (IST-1999-10079) and by the Italian Health Ministry.

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