Amygdala input promotes the spread of excitatory neural activity from the perirhinal cortex to the entorhinal/hippocampal neurocircuit

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ABSTRACT

A number of sensory modalities most likely converge in the rat perirhinal cortex. The perirhinal cortex also interconnects with the amygdala, which plays an important role in various motivational and emotional behaviors. The neural pathway from the perirhinal cortex to the entorhinal cortex is considered one of the main paths into the entorhinal-hippocampal network, which has a crucial role in memory processes. To investigate the potential associative function of the perirhinal cortex with respect to sensory and motivational stimuli and the influence of the association on the perirhinal/entorhinal/hippocampal neurocircuit, we prepared rat brain slices including the perirhinal cortex, entorhinal cortex, hippocampal formation, and amygdala. We used an optical imaging technique with a voltage-sensitive dye to analyze (1) the spatial and functional distribution of inputs from the lateral nucleus of the amygdala to the perirhinal cortex, (2) the spread of neural activity in the perirhinal cortex after layers II/III stimulation, which mimics sensory input to the perirhinal cortex, and (3) the effect of associative inputs to the perirhinal cortex from both the lateral amygdaloid nucleus and layers II/III of the perirhinal cortex on the perirhinal-entorhinal-hippocampal neurocircuit. Following stimulation in the superficial layers of the perirhinal cortex, electrical activity only propagated into the entorhinal cortex when sufficient activation occurred in the deep layers of perirhinal area 35. We observed that single stimulation of either the perirhinal cortex or amygdala did not result in sufficient neural activation of the deep layers of areas 35 to provoke activity propagation into the entorhinal cortex. However, the deep layers of area 35 were depolarized much more strongly when the two stimuli were applied simultaneously, resulting in spreading activation in the entorhinal cortex. Our observations suggest that a functional neural basis for the association of higher-order sensory inputs and emotion-related inputs exists in the perirhinal cortex, and that transfer of sensory information to the entorhinal-hippocampal circuitry might be affected by the association of that information with incoming information from the amygdala.
Key words: Perirhinal cortex; Entorhinal cortex; Amygdaloid nucleus; Association; Optical imaging; Voltage-sensitive dye
INTRODUCTION

The perirhinal cortex is thought to be a crucial part of the medial temporal lobe declarative memory system, not only as a relay point for the information stream from the sensory cortices to the hippocampal formation, but also as an associative area for many kinds of information. In the hippocampal memory system (Squire and Zola-Morgan 1991), the pathway from the perirhinal cortex to the entorhinal cortex is regarded as one of the main paths into the entorhinal-hippocampal network, which is crucially involved in memory formation (Burwell et al. 1995; Suzuki 1996; Witter 1993). The significance of the perirhinal cortex in memory processing has been tested by assessing the behavioral effects of specific lesions. (Meunier et al. 1993). (Corodimas and LeDoux 1995; Wiig and Bilkey 1995). In the rat, the perirhinal cortex receives inputs from somatosensory, auditory, visual, and olfactory association areas (Burwell 2001; Burwell et al. 1995; Suzuki 1996). The perirhinal cortex projects robustly to the lateral entorhinal cortex, although these afferents provide up to 16% of its cortical input, and are thus relatively less strong in the rat than they are in the monkey (Burwell and Amaral 1998b, 1998a). Notwithstanding these rather elaborate studies of the structure and significance of the perirhinal cortex, only a few studies have addressed the connections of the perirhinal cortex with the entorhinal-hippocampal system, including one using electrophysiological methods in the rat in vivo (Naber et al. 1999) and two in guinea pig isolated whole brain (Biella et al. 2001; Biella et al. 2000).

The perirhinal cortex is also thought to be involved in emotional memory, in view of the prominent interconnections of the perirhinal cortex with the amygdala, which is considered as an emotion-related area (Cahill et al. 1995; LeDoux 1987; Ono et al. 1995). In monkeys, the polar portion of the perirhinal cortex has powerful and reciprocal connections with a number of amygdaloid nuclei, including the lateral, basal, and accessory basal nuclei (Suzuki 1996). In rats, the perirhinal cortex has its strongest interconnections with the lateral nucleus, although minor reciprocal connections with the accessory basal nucleus have also been described.
(Burwell et al. 1995; Krettek and Price 1977a, 1977b; Suzuki 1996). Herzog and Otto (Herzog and Otto 1998) found that lesions limited to the perirhinal cortex attenuated the expression of fear related to an olfactory conditioning stimulus, but not to the training context, and suggested that the perirhinal cortex is a critically important component of the neural system, mediating the acquisition or expression of associations between olfactory and foot-shock inputs. Likewise, lesions of the perirhinal cortex interfere with the expression of conditioned fear responses to visual (Rosen et al. 1992), auditory (Campeau and Davis 1995), and contextual stimuli (Corodimas and LeDoux 1995). Moreover, in electrophysiological experiments in anesthetized cats, Collins et al. (Collins et al. 2001) recorded spontaneous activity dominated by a slow focal oscillation at 1 Hz onto which a faster rhythm (~ 30 Hz) was superimposed in both the perirhinal cortex and the amygdala; they suggested that the perirhinal cortex and the amygdala are functionally coupled. In spite of the many efforts to study the relationship between the perirhinal cortex and amygdala, the functional organization between the two is still poorly understood. To investigate functional organization of the brain, optical imaging of neural activity with a voltage sensitive dye is a powerful technique. By using voltage sensitive dyes, neural activation and pathways in brain slices were successfully monitored (Barish et al. 1996; Grinvald et al. 1982; Iijima et al. 1996; Jackson and Scharfman 1996; Tanifuji et al. 1994; Tominaga et al. 2000; Yuste et al. 1997). Here, we describe a functional connection between the perirhinal cortex and the entorhinal-hippocampal circuitry, more specifically, the influence of amygdala stimulation on perirhinal-entorhinal neural propagation. For this study, we obtained rat brain slices including the perirhinal cortex, entorhinal cortex, hippocampal formation, and lateral amygdaloid nucleus. These sections were used for in vitro experiments, in which we used an optical imaging technique with a voltage-sensitive dye to analyze the spatio-temporal distribution of excitatory activity elicited by separate and combined stimulation to the perirhinal cortex and amygdala.
MATERIALS & METHODS

Slice preparation and Solutions

Male wistar rats (100~160g) were deeply anesthetized by ether and decapitated. Slices (400µm thickness) were prepared using a vibratome (DTK 3000W, Osaka, Japan) in ice-cold sucrose artificial cerebrospinal fluid (s-ACSF) (Moyer and Brown 1998). The s-ACSF was composed of 248 mM sucrose, 5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 2 mM MgSO$_4$, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 22 mM NaHCO$_3$, 10 mM D-glucose, and oxygenated with a mixture of 95%O$_2$ 5%CO$_2$, pH 7.4 chilled to 4°C. The slices including the perirhinal cortex (PC), entorhinal cortex (EC), hippocampal formation, and lateral amygdaloid nucleus (AMG) were cut at an angle indicated by the line “h” shown in Fig.1A. For this, the hemispheres were separated and their dorsal part removed by razor cuts parallel to the line indicated by “h”, and each hemisphere was glued with its dorsal cut surface to a vibratome stage. Fig.1C shows an example of a nissl-stained slice cut at the level of line “h”. With the use of such slices, we investigated propagation of neural activity following stimulation of perirhinal layers II/III, and of amygdala.

The known anatomical neural connectivity in such slices is illustrated as a simplified block diagram in Fig.1B. In some experiments we used slices cut at approximately 45°, as indicated by the line marked “a” in figure 1A. To prepare such slices, first the rostral and caudal parts of the hemisphere were removed by razor cuts parallel to line “a”, and the remaining portion of the hemisphere was glued with its rostral cut-surface to a vibratome stage. An example of a nissl-stained slice cut at this angle is shown in Fig.1D. Such angled slices have the benefit that the perirhinal-entorhinal border can be easily identified. We used also this type of slices for investigating the perirhinal-entorhinal pattern of spreading activity and to verify whether the presence of a physiological connection between perirhinal and entorhinal cortices depended on the angulation of the slices. With the use of the lipophylic tracer DiI (1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate) (Gelperin and Flores 1997; Godement et al. 1987), we verified the existence of connectivity between the perirhinal
and entorhinal cortices in slices cut at the angle “h” and “a”, and between amygdala and
perirhinal cortex in slices cut at the angle “h”.

Before starting recording, slices were submerged in the incubation chamber with standard
ACSF for more than one hour. The standard ACSF used for incubation and recording consists
of 124 mM NaCl, 5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 2 mM MgSO$_4$, 2 mM CaCl$_2$, 22 mM NaHCO$_3$,
10 mM D-glucose, oxygenated with a mixture of 95%O$_2$ 5%CO$_2$ (pH 7.4). The ACSF was
maintained at room temperature (26~28°C).

**Optical recording system and staining with voltage-sensitive dye**

The optical recording methods were similar to those reported elsewhere (Barish et al. 1996;
Iijima et al. 1996). For recording, a slice was submerged in a recording chamber under the
tandem type microscope, which was constructed in our laboratory (Iijima et al. 1996;
Takashima et al. 1999). We used a 50 mm Nikon camera lens (F1.25) as the first objective,
and a 105 mm camera lens (F1.85) for the second objective, which altogether results in
approximately a two fold magnification. This optical apparatus allowed us to record from
approximately a 5 × 5 mm area of the brain slice. The slice was stained in the recording
chamber for 3 minutes with the voltage-sensitive dye RH-795 (0.5mg/ml ACSF). The
voltage-sensitive dye has excitation and emission maxima at 530 nm and 712 nm, respectively.
Changes in fluorescence of the dye decrease proportional to the changes of membrane
depolarization. After the staining, extra dye was washed out with oxygenated superfusion
solution and the preparation was incubated in the recording chamber for another 10 ~ 15
minutes before the optical measurements were performed. The stimulus-evoked responses
were recorded as fractional changes of fluorescence by the image processor with the use of a
MOS-based camera, developed in our laboratory (Ichikawa et al. 1993). Illumination from a
tungsten–halogen lamp was passed through a heat filter (less than 10% transmittance for 700
~ 1600 nm light). The excitation light filtered at 535 nm (±20 nm bandpass) was reflected down
onto the preparations by a dichroic mirror (half-reflectance wave length of 580 nm). The
fluorescence from the preparation was projected to the image sensor through a long-wavelength pass filter (50% transmittance at 600 nm). The fractional changes were detected and stored in the frame memory of the imaging system connected to a PC/AT computer. The resolution of the MOS-image sensor was 128 x 128 pixels. To improve the signal to noise ratio, we binned 2 x 2 pixels into one pixel, and thus images are represented as 64 x 64 pixels. By using this optical imaging system, we acquired the 500 or 1000 frames by the rate of 0.6, 1.2 or 2.4 ms/frame. To represent the spread of neural activity, we superimposed color-coded optical signals on the bright field image of the slice. In this procedure, we applied a color code to the fraction of the optical signal, which exceeded the baseline noise. That is, optical signals whose sizes were close to the baseline noise were ignored. For reducing the base line noise, we sometimes averaged four identical recordings acquired with a 5 sec. interval. The data set was averaged in the frame memory directly. The optical signal was analyzed offline using custom-made software or Origin software (OriginLab Corp., MA, USA). Recordings were made in a total of 58 slices.

**Field potential recording**

A glass recording electrode filled with normal medium (5–10 MΩ) was positioned in the part of perirhinal layers II/III under the rhinal sulcus. The field potential was filtered at 1kHz low-pass filter, amplified (MEZ-8300, Nihonkoden, Japan), and digitized with the use of the optical imaging system through its external input terminal. The field potential was continuously monitored throughout the experiment, and no photo dynamic effect on its amplitude was observed by the optical recording.

**Electrical stimulation to the Perirhinal Cortex and the Amygdala**

The stimulating electrode was a tungsten bipolar electrode with a tip separation of 150 µm. Since anatomical studies indicate that layers II/III of the perirhinal cortex receive projections
from sensory areas (Burwell and Amaral 1998b; Faulkner and Brown 1999), we placed the stimulus electrode for the perirhinal cortex on layers II/III. Initially, a single pulse electrical stimulation was applied. To mimic input from the sensory cortices to the perirhinal cortex in a brain slice, we stimulated an extensive area of layers II/III of perirhinal cortex using a stimulus intensity which evokes maximum response in perirhinal cortex. In Fig 1C, electrical and optical signals extracted from layers II/III of perirhinal cortex are shown following different stimulus intensities ranging from 40 to 100 µA. The stimulus intensity and duration that evoked the maximum response in layers II/III of the perirhinal cortex was 80-100 µA and 300 µs, respectively. This set of parameters was chosen for all subsequent experiments. Similarly, we established the optimal protocol for amygdala stimulation. We needed to stimulate the amygdala repeatedly (40 Hz) with 100~150 µA for 300 µs.

**Drugs**

The entorhinal cortex has been reported to harbor many more cells containing the calcium-binding protein parvalbumin than is the case for the perirhinal cortex. Since it has been well established in these areas that parvalbumine colocalize for 100% with γ-aminobutyric acid (GABA), it is likely that in the entorhinal cortex more GABA-containing cells will be present compared to the perirhinal cortex (Burwell et al. 1995; Miettinen et al. 1996). To investigate the spatio-temporal distribution of excitatory activities between the perirhinal cortex and the entorhinal-hippocampal circuit, synaptic inhibition was partly suppressed by applying a low concentration of the GABA_A antagonist bicuculline (1~2µM) ((-)bicuculline methiodide, Sigma-Aldrich Co., MO) in the recording solution. In such condition, our optical imaging system has already measured the entorhinal-hippocampal excitatory neural spread elicited by the electrical stimulation to the lateral entorhinal cortex in rat brain slices without inducing spontaneous paroxysmal activities (Iijima et al. 1996). Under similar conditions, it is expected that the neural spread of excitatory activities from the perirhinal cortex to the entorhinal cortex might be also observed following electrical stimulation to the
In some experiments, the neural spreads of excitatory activities from the perirhinal to the entorhinal cortices were investigated by increasing the concentration of bicuculline (5µM, and 10µM). Under 10µM concentration, spontaneous synchronous excitatory activities were recorded by glass electrode, whereas with 5µM of bicuculline no spontaneous activity was recorded. We measured the optical signals related to evoked response only in those conditions during which no spontaneous activity was recorded by the glass electrode.

**Histology**

The brain slices were fixed with 4% paraformaldehyde for more than one week. Subsequently, the slices were dipped in PBS with 30% sucrose for more than 10 hours, and cut at 70µm thickness with the use of a freezing microtome (SM-2000R, Leicamicrosystems). Sections were nissl-stained by 0.25% thionin solution. Fig.1C and D shows the histological result. The areas of the perirhinal and entorhinal cortices were delineated on the bases of cytoarchitectural criteria (Burwell et al. 1995). By comparing these histological data with the optical imaging data, we could identify the area in which neural propagation was recorded.
RESULTS

**Associative stimuli to the Perirhinal Cortex and the Amygdala**

Initial data were collected from 23 slices kept in ACSF with 1~2 µM BMI. A representative experiment is illustrated in Figure 2. The traces in Fig. 2A represent changes in the optical signal over time, recorded from selected points indicated in Fig. 2B (AMG; lateral amygdaloid nucleus, PC; perirhinal cortex, MEC; medial entorhinal cortex, DG; dentate gyrus). Figure 2C shows enlarged traces of perirhinal layers II/III and area 35 in Fig. 2A. In the case of a single stimulus in the perirhinal cortex (indicated in Fig. 2A, left column, below), a response in part of the perirhinal cortex was evoked 5 ms after the stimulation, and the amplitude of the response became maximal at 40 ms. In this case, no changes in the optical signal were observed in the medial entorhinal cortex or dentate gyrus, but a small response was observed in the amygdala (Fig. 2B, left column). Figure 2B (middle column) shows the responses evoked by repetitive stimulation of the amygdala (5 pulses at 40 Hz; indicated in the lowest time chart). The activity in the amygdala increased during the stimulation period. Activity in the deep layers of the perirhinal cortex was evoked 10 ms after the stimulation was applied, and increased slowly until 65 ms. As with perirhinal stimulation, amygdala stimulation did not evoke responses in the medial entorhinal cortex or dentate gyrus. When the perirhinal cortex and the amygdala were stimulated simultaneously, however, responses corresponding to a 0.02% fractional change in the medial entorhinal cortex and dentate gyrus were evoked (Fig. 2B, right column). Note that in this case, the trace for the deep layers of area 35 in the perirhinal cortex indicates enhanced neural activity (marked by a red asterisk in the red trace), which was caused by the association of both the amygdala and perirhinal cortex stimuli, whereas the responses in layers II/III of area 36 were suppressed (marked by a red arrow in the red trace) compared to the result of the amygdala stimulation (marked by a black arrow). The respective latencies of the changes in activity in the medial entorhinal cortex and dentate gyrus were 100.8 and 104.8 ms after the stimulation was applied.
Real time imaging of these results is shown in Fig. 2D. The evoked responses in perirhinal layers II/III elicited by the perirhinal stimulation increase until the image acquired at 48 ms. Subsequently, the neural responses decrease (Fig. 2D-a). In the middle row (Fig. 2D-b), the activity in the perirhinal cortex, evoked by the amygdala stimulation, is maximal in the 60-ms frame. The neural responses decrease after 60 ms, in spite of applying the stimulus at 40 Hz continuously. In the results shown in (a) and (b), there are fractional changes of about 0.01% in the deep layers part on the border of the perirhinal-entorhinal cortices, but no responses are observed in the medial entorhinal cortex or hippocampal formation. In Fig. 2D-(c), we illustrate the effect on the perirhinal/entorhinal/hippocampal network of combined stimulation of both the amygdala and layers II/III of the perirhinal cortex. Combined stimulation evokes much greater signal changes in the deep layers than when only one site is stimulated, especially in area 35 at 48 and 60 ms, although the responses in layers II/III of the perirhinal cortex are less than in the images at 60, and 96 ms following amygdala stimulation. The activity in the deep layers of area 35 is followed by responses in all layers of the lateral entorhinal cortex at 60 ms. This evoked activity appears to propagate medially (to 96 ms), and responses are observed in the dentate gyrus of the hippocampal formation at 132 ms. Similar observations were obtained in 13 of the 23 slices. Similar experiments were carried out in six slices without bicuculline. Although the extent of the spread of activity in the horizontal and vertical directions in the perirhinal cortex was not strongly affected, evoked potentials were shorter and of smaller amplitude compared to the 1-2 µM bicuculline condition. Without bicuculline, combined stimulation produced similar effects in area 35 and adjacent parts of the lateral entorhinal cortex, although extensive medial spread of excitation followed by evoked hippocampal activities was not observed (not illustrated).

**Responses elicited by repetitive stimulus**

Since it is generally accepted that the perirhinal cortex is one of the major sources of multimodal cortical input for the hippocampal system, the finding that single stimulation of the
perirhinal cortex does not evoke activity in either the entorhinal cortex or the hippocampal formation merits further investigation. In 21 slices bathed in ACSF with 1-2 µM bicuculline, we tested whether repetitive stimulation of the perirhinal cortex leads to activation of the entorhinal cortex. This was combined with repetitive amygdala stimulation. A representative experiment is illustrated in Figure 3, which shows that no entorhinal-hippocampal neural activity was evoked under all experimental conditions in spite of repeated stimulation of the perirhinal cortex or the amygdala. In Fig. 3A, the responses elicited by a stimulus electrode in the superficial layers of the perirhinal cortex are mainly propagated in the rostrocaudal direction within the stimulated superficial layers until 19.2 ms. The activity can be seen to spread to the deep layers of the perirhinal cortex in the images taken from 19.2 to 28.8 ms. Responses encoded in red, which represents the peak value of the fractional change in this acquisition, are prominent in layers II/III at 43.2 ms. Irrespective of the stimulation protocol used, the overall features of the observed pattern of perirhinal activation were similar (Fig. 3A-b, and -c).

As seen from Fig. 3C (upper panel), repetitive stimulation did not lead to marked increases in the amplitude of the neural response in layers II (no increase) or V (~10% increase), but the onset of recorded activation was delayed slightly. The evoked neural responses reached the steady state during acquisition, and no seizure activity developed in any case. In the three experimental situations, we never observed activity in the entorhinal cortex or hippocampal formation, even when we applied repetitive stimulation at 40 Hz (Fig. 3C, upper panel, LEC trace).

Figure 3B shows the propagation pattern after the amygdala stimulation. Fig. 3Ba and b shows that both single and repetitive stimulation resulted in a restricted activation increase in a single spot in the deep layers of the perirhinal cortex, with subsequent activation of the superficial layers. This pattern is fairly stable up until 24 ms. After this time, neural activation propagates to an extensive area of the perirhinal cortex, from the deep layers to the superficial layers. Comparing the repetitive stimulation image at 38.4 ms with the single pulse result, the neural responses are increased, especially in the deep layer and middle layer parts of the
perirhinal cortex, which show much higher responses. The depolarization of layers II/III reached a peak at 67.2 ms. With amygdala stimulation, we recorded the same or higher amplitudes of neural activity in the perirhinal cortex than following perirhinal stimulation. Figure 3C (lower panel) shows examples of optical signal traces extracted from layers II/III, layers V/VI of perirhinal cortex (PC), the lateral entorhinal cortex (LEC), and the amygdala (AMG). The neural activity in the amygdala increases during periods of repetitive stimulation. In the perirhinal cortex, although repetitive stimuli yield larger amplitude responses compared to single pulse stimulation, the profiles of traces following 5- and 10-pulse stimulation are very similar. Despite this increased activity, we never observed lasting depolarization (total of 21 slices). Based on these results, the 5-pulse stimulus at 40 Hz was considered the optimal stimulus for perirhinal activation.

Stimulation of the deep layers of the Perirhinal Cortex near the Perirhinal/Entorhinal border

The results of single stimulation of the perirhinal cortex with or without co-stimulation of the amygdala (Fig. 2) indicate that sufficient neural activation in the deep layers near the perirhinal/entorhinal border is essential for subsequent activation of the lateral entorhinal cortex. In a subsequent series of experiments, we analyzed the relevance of activation in the deep layers of the perirhinal cortex in more detail. Figure 4 illustrates that activation of the deep layers of area 35 in the perirhinal cortex is important for the neural spread from the perirhinal to the entorhinal cortices. The slice was cut at the angle shown in Fig. 1A, parallel to “a”. To elucidate the physiological connections from the perirhinal to the entorhinal cortices, we initially cut slices at various angles. By cutting at angle “a”, we obtain brain slices in which the connectivity of interest is maintained and in which the boundary of the perirhinal cortex and the lateral entorhinal cortex can be distinguished easily (Burwell et al. 1995). A Nissl-stained section is shown in Fig. 4B. The perirhinal area was divided into areas 35, and 36 based on
differences in cytoarchitecture. Optical measurements were performed in the rectangular area shown in this figure. We applied bicuculline at a concentration of 2 µM. The neural responses elicited by stimulation of the superficial, middle, or deep layers parts of area 36, at the border with area 35, were measured. Repetitive stimulation at 40 Hz to the superficial or middle layers evoked neural activity that propagated to an extensive area of the perirhinal cortex and temporal cortex, but neural activity was not initiated in the lateral entorhinal cortex (Fig. 4A-ab). A single pulse stimulation to the deep layers evoked neural activity in area 35 and a small response reached the area-35/LEC border (24-ms image in Fig. 4A-c). Repetitive stimulation of the deep layers caused much higher responses in area 35 of the perirhinal cortex and in the lateral entorhinal cortex near the perirhinal /entorhinal border (72-ms image in Fig. 4A-d), spreading in the direction of the medial entorhinal cortex (Fig. 4A,d 72-216 ms). Fig. 4B illustrates the time course of optical signals reflecting the imaging results shown in the two lower rows in Fig. 4A, i.e., single and repetitive stimulation of the deep layers of perirhinal cortex. The traces extracted from area 35 in the perirhinal cortex indicate that the neural response evoked by repetitive stimulation in this area increased slowly (~150 ms), until the amplitude was approximately doubled (shown as the dotted area in Fig. 4B, trace number 2). This increasing depolarization of area 35 propagated medially into the entorhinal cortex. This set of experiments was performed on 28 slices. The results obtained in slices taken at different rostral to caudal levels all had similar features (we did not use slices including the postrhinal cortex). Moreover, we obtained similar results when slices were recorded in the presence of 5µM bicuculline (n=20).
DISCUSSION

This study examined the propagation of perirhinal evoked activity into the entorhinal cortex and the associative effect of amygdala input. The main findings can be summarized as follows. First, propagation of activity from the perirhinal to the entorhinal cortices requires sufficient neural activation in the deep layers near the perirhinal/entorhinal border (area35). Second, stimulation of the superficial part of the perirhinal cortex, which receives many projections from other cortical areas, alone does not evoke sufficient activity to initiate the entorhinal response from area 35. Third, the perirhinal deep layers can be activated by repeated stimulus of the lateral amygdaloid nucleus, and combined stimulation of both the perirhinal cortex and the amygdala evoked much higher depolarization in the deep layers of the area 35, leading to initiation of propagation from the perirhinal cortex into the entorhinal-hippocampal circuit.

Rat perirhinal and entorhinal connections have been reported in detail (Burwell and Amaral 1998a; Burwell et al. 1995; Naber et al. 1997). On the other hand, the physiological correlates of such connectivity have not been investigated adequately. This study used optical recordings to obtain data about the physiological characteristics of the neural connectivity between the perirhinal cortex and the entorhinal cortex. It has been suggested that area 35 of the perirhinal cortex projects strongly to the lateral part of the entorhinal cortex, and that these projections to the entorhinal cortex originate in layers III and V and terminate preferentially in layers II and III (Burwell and Amaral 1998a; Burwell et al. 1995). Naber et al. reported that anterogradely labeled fibers from the perirhinal cortex are widely distributed in the lateral entorhinal cortex, in both the superficial and deep layers. In addition, they indicated that injections which selectively involved the deep layers of the perirhinal cortex produced more labeled fibers in the lateral entorhinal cortex than injections in superficial layers (Naber et al. 1997). Our results indicate that the perirhinal deep layers are essential for the propagation of excitatory activity from the perirhinal cortex to the lateral entorhinal cortex. Moreover, our finding that only stimulation to the deep layers evoked sufficient neural activity in area 35 to initiate perirhinal-entorhinal
excitation propagation supports the anatomical findings.

In our experiment, inhibition was partly suppressed with the application of a low concentration of bicuculline. However, even under such conditions, neural activity in the lateral entorhinal cortex was not evoked by either perirhinal superficial layers stimulation or amygdala stimulation alone. If the anatomical organization of the perirhinal-to-entorhinal connections is taken into consideration, the following questions arise. First, was the angle of the slice that we used suitable? Our results were obtained using several differently angled slices (horizontal and 45° slices (this study), and coronal slices (Iijima et al. 1994)). Irrespective of the angle of the slice, comparable observations were collected. Therefore, the angle of the slice does not appear to be critical. Second, are sufficient projection fibers to the entorhinal cortex included in a slice of 400 µm thick? Although it cannot be denied that some projection fibers to the lateral entorhinal cortex will be severed or removed in such a slice preparation, anatomical data indicate that such connections will be sufficiently preserved (Burwell and Amaral 1998a; Naber et al. 1999). In fact, the preservation of the relevant circuitry has been confirmed by us using Dil-crystal placement in relevant parts of the slice. Moreover, a recent experiment using preparations of the guinea pig whole brain (Biella et al. 2001; Biella et al. 2000) also demonstrated very weak direct perirhinal-to-entorhinal connectivity with physiological methods, and the connections that were observed are mediated by polysynaptic circuits. In that study, stimulation of area 36 induced activation within area 36, but not in the EC. In addition, we obtained similar data in a preliminary study using an optical imaging technique with the guinea-pig whole brain. These observations strongly suggest that our results concerning the difficulty of activating the entorhinal cortex by perirhinal stimulation is not specific to brain slices. These considerations suggest that the neural architecture in the perirhinal/entorhinal border area blocks propagation from the perirhinal to the entorhinal cortex. This blockade could involve a strong inhibitory system in area 35, because a high concentration of bicuculline appeared to induce neural spread from the perirhinal cortex to the lateral entorhinal cortex (data not shown). In addition, the results reported here (shown with the
dotted circle in the red trace of Fig. 5C) seem to indicate that neurons in area 35 are not easily depolarized to the level, which causes propagation of activity from the perirhinal to the entorhinal cortices. This feature might be the result of a polysynaptic structural feature or a synaptic delay resulting from the membrane electrical properties of neurons in this area. Perirhinal layers II/III (Beggs et al. 2000; Faulkner and Brown 1999), VI (Faulkner and Brown 1999), and V (Moyer et al. 2000) contain many late-spiking neurons, which characteristically possess slowly inactivating potassium channels. Such a neuron might have delayed and sustained firing properties in response to long synaptic trains. It has been suggested that these cells show prolonged synaptic integration, and are involved in tonic neural activation measured in rat perirhinal neurons during the delay period in an odor-guided, delayed nonmatching-to-sample task (Young et al. 1997).

Another explanation for this apparent perirhinal-entorhinal gating mechanism is that neurons in the perirhinal cortex have a structural feature particularly suitable to associate incoming inputs from different origins. Indeed, the perirhinal cortex is thought to be a zone of convergence from higher order sensory association areas and a number of different subcortical structures (Suzuki 1996). That is, perirhinal cells which project to entorhinal cortex and/or their pre-synaptic cells in perirhinal cortex should receive input from many areas in the brain. In other words, stimulation of a restricted perirhinal area might leave many non-depolarized fibers. In fact, we showed that stimulation of an extensive area of perirhinal layers II/III at maximum stimulus intensity (Fig. 3A) or stimulation of two sites in perirhinal layers II/III (data not shown) did not induce sufficient neural activity in the deep layers for propagation to the entorhinal cortex to occur. In contrast, perirhinal stimulation associated with repetitive stimulation of the amygdala evoked a much stronger response in the deep-layer part of the perirhinal/entorhinal border than perirhinal stimulation alone. These results suggest that some neurons in the deep layers that were evoked by perirhinal/amygdala associative stimulation were not evoked by perirhinal stimulation alone.

Stimulation of amygdala by itself undoubtedly evoked neural responses in the perirhinal
cortex (Fig. 3B). This result confirms the anatomical observation that fibers from the amygdala terminate in perirhinal layers V and III (Krettek and Price 1977b). The responses observed in layers II/III should result from complex monosynaptic responses of the amygdala and polysynaptic responses in the perirhinal cortex. Our results also showed that repetitive stimulation evoked much higher depolarization (~40% increase) in perirhinal neurons in the deep and superficial layers. Lisman suggested that the best stimulus for exciting a cell (that is, a neural code) consists of brief bursts of high-frequency firing (Lisman 1997). However, excess repetitive stimuli were not effective to result in neural depolarization in perirhinal neurons. Consequently, as the maximum stimulus for our experiments, we used 5 pulses at 40 Hz to stimulate the amygdala. In our experiments, continuous stimuli to the perirhinal cortex or the amygdala were not effective in producing long-lasting perirhinal depolarization. In addition, we showed that associative stimuli to both areas suppressed the neural responses delivered from the amygdala in layers II/III of the perirhinal cortex (the black to the red arrows in Fig. 2A), and enhanced the responses in the deep layers (red asterisk in Fig.2A). This post-synaptic response in the perirhinal cortex suggests that the excitability of perirhinal neurons was increased by associative or repetitive inputs, which arrive during the early phase (~75ms), but not during the decay phase. Moreover, the enhanced neural activity in the perirhinal deep layers resulting from associative stimuli to the perirhinal cortex and amygdala was only observed when both stimuli were applied during an ~100 ms time-window (data not shown).

We postulate that one of the mechanisms initiating propagation from the perirhinal cortex to the entorhinal cortex is through emotion-related inputs from the amygdala that become associated with higher order sensory inputs to the perirhinal cortex, triggering neural propagation from the perirhinal to the entorhinal cortices. It might be also possible that direct thalamo-perirhinal inputs to the perirhinal cortex (Guldin and Markowitsch 1983; Wouterlood et al. 1990) play an additional role. It has been suggested that the dichotomy between the roles of the amygdala and the perirhinal cortex is not absolute. For example, perirhinal lesions performed after classical fear conditioning reduce or abolish conditioned fear responses
(Corodimas and LeDoux 1995; Rosen et al. 1992). In addition, much data suggest that the AMG modulates the neural processes involved in the formation of declarative memory (Cahill 2000; Cahill and McGaugh 1998). For instance, long-term explicit memory of emotionally arousing stories is enhanced compared with memories of neutral stories, and lesions of amygdala abolish this effect (Adolphs et al. 1997; Cahill et al. 1995). Moreover, brain-imaging studies have found a high correlation between long-term recall of emotionally arousing or neutral material and the amount of amygdala activation observed when these stimuli were first presented (Cahill 1996; Hamann et al. 1999). Currently, it is unclear how the amygdala modulates declarative memory. The reciprocal amygdala-perirhinal connections, in addition to the amygdala-hippocampal connections (Krettek and Price 1977a, 1977b; Russchen 1982) constitute an obvious possibility, but little is known of their physiology. The present results are in line with the idea that amygdala-perirhinal connections are critically involved, showing that stimulation of the perirhinal cortex, associated with amygdala stimulation evoked neural activity in the entorhinal cortex, and this activity subsequently propagated into dentate gyrus, whereas stimulation of either perirhinal cortex or amygdala alone did not evoke responses in the entorhinal cortex nor in the dentate gyrus.

In conclusion, the optical signal traces extracted from the deep layers of perirhinal cortex indicate that perirhinal deep-layer cells are activated by simultaneous associative stimuli to both the amygdala and perirhinal cortex. Needless to say, we cannot conclude that inputs from sensory cortices and inputs from the amygdala terminate on a single perirhinal neuron associatively, because our results are not obtained from single-cell-level recordings. However, the fact that the neural activation evoked in the perirhinal cortex propagated to the entorhinal-hippocampal-circuit only in case of associative stimulation to the perirhinal cortex and the amygdala suggests that the association of higher-order sensory inputs and emotion-related inputs could take place in the perirhinal cortex. Moreover, it appears likely that information transfer from the perirhinal cortex to the entorhinal-hippocampal-circuit is strongly dependent on the association of that information with concurrent activation of the amygdala.
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FIGURE LEGENDS

Figure 1. Preparation of rat brain slices and relationship between electrical and optical signals.

A. Lateral view of the rat brain indicating the position of the perirhinal cortex (areas 35 and 36) to either side of the rhinal sulcus. Approximate orientations of the in vitro slices are illustrated by “h” and “a” lines, respectively. B. A simplified block diagram of the known neural connectivity of the rat perirhinal cortex. C. A Nissl-stained slice cut at the angle “h” in Fig.A. This type of slice includes the hippocampus, entorhinal cortex, perirhinal cortex, and lateral amygdaloid nucleus. MEC: medial entorhinal cortex, LEC: lateral entorhinal cortex, PC: perirhinal cortex, AMG: lateral amygdaloid nucleus. The rhinal sulcus (rs) indicates the border between the entorhinal and perirhinal cortices. The optical recording was performed in the area indicated by the rectangle. D. A Nissl-stained slice cut at the angle “a” in Fig.A. The boundary of the perirhinal cortex and lateral entorhinal cortex can be distinguished easily. By use of this type of slices, spread of excitatory neural activity from the perirhinal cortex to the entorhinal cortex was investigated. E. Relationship between electrical and optical signals evoked by perirhinal stimulation in a slice. The traces elicited by various stimulus intensities were measured in layers II/III of the perirhinal cortex near the rhinal sulcus (rs).

Figure 2. Optical recording of associative inputs in the perirhinal cortex

A. Matrix of optical signals indicating the responses elicited by stimulation of the perirhinal cortex (PC), lateral amygdaloid nucleus (AMG), or both areas. For the amygdala stimulation, repeated electrical stimulation (5 pulses at 40 Hz) was applied as indicated below. Associative stimulation of both the perirhinal cortex and the amygdala resulted in a (asterisk) response in the deep layers of the perirhinal cortex (area 35), while there was no similar response in layers II/III (parallel between black and red arrows). B. Schematic drawing of a brain slice showing
the lateral amygdaloid nucleus (AMG), perirhinal layers II/III (PC II/III), area 35 of perirhinal cortex, medial entorhinal cortex (MEC), and dentate gyrus (DG).  

C. The initial phase of the depolarization on an expanded time scale. Left and right traces were extracted from perirhinal layers II/III and Perirhinal area 35 shown in B, respectively. The red traces indicate the responses elicited by associative stimulation of both the perirhinal cortex and the amygdala. The blue and green traces correspond to the responses elicited by the stimulation of perirhinal cortex or amygdala alone, respectively. 

D. Series of selected images taken at different time intervals after the electrical stimulation (time indicated below each image). Depolarization is measured as the fractional changes in fluorescence in each pixel, this value is encoded in “pseudocolor” as indicated in the scale, and is superimposed on a bright-field image of the slice.  

(a) The neural propagation pattern elicited by a single pulse stimulation to layers II/III in the perirhinal cortex (PC).  
(b) The result of amygdala (AMG) stimulation (5 pulses at 40 Hz).  
(c) The result of associative stimulation to both the perirhinal cortex (PC) and amygdala (AMG).  

Single pulse stimulation of the perirhinal cortex with 5-pulse stimulation at 40 Hz evoked entorhinal-hippocampal neural activation.

Figure 3. Optical recording of perirhinal neural activity elicited by stimulation of the Perirhinal Cortex or Amygdala alone

These results were obtained from the same slice shown in Fig. 2. The recording area and stimulation site are shown in Fig. 2B.  

A. Time-series of images illustrating the distribution of activity evoked by electrical stimulation of the perirhinal cortex with 1, 5, and 10 pulses at 40 Hz. The time after the electrical stimulation is indicated below each image.  

B. Time-series of images illustrating the distribution of activity evoked by electrical stimulation to the amygdala with 1 and 5 pulses at 40 Hz.  

C. Time course of the optical signals extracted from part of perirhinal layers II/III (PC II/III), perirhinal layers V/VI (PC V/VI), the lateral entorhinal cortex (LEC), and the amygdala (AMG). The upper and lower traces show the results of perirhinal cortex and amygdala stimulus experiments, respectively. The stimulus timing is shown above
each set of traces. In every case, there is no neural activity in the entorhinal cortex, or hippocampus. We applied a low concentration of bicuculline (2µM); in none of the conditions we observed seizure activities.

Figure 4. Optical recordings showing that stimulation of the perirhinal deep layers is effective for neural propagation to the entorhinal cortex from the perirhinal cortex

A. Time-series of images illustrating the distribution of activity in the different stimulation conditions. The brain slice cut along the “a” line in Fig. 1A was used. The optical recording was made in the area enclosed by the dotted rectangle on the nissl-stained slice in Fig.4B. The stimulus electrode was placed at the superficial, middle, and deep layers of the perirhinal cortex. The stimulus site and stimulus frequency are indicated above each series. Blue dots on the first images (0 ms image) mark the stimulation sites of the bipolar electrode. On some images, the morphological features were superimposed. Perirhinal–entorhinal neural spread was only observed when deep layers were stimulated repetitively; single-pulse stimulation was not effective. B. The time course of optical signals elicited by stimulation of the deep layers of the perirhinal cortex. In the traces extracted from area 35, the depolarization was increased slowly by repetitive stimulation (shown by the dotted circle on the red trace). These data were obtained with the low concentration of bicuculline (2µM). PC; perirhinal cortex, EC entorhinal cortex, TE; temporal cortex
Figure 1

A

B

Hippocampal formation

Entorhinal Cortex (EC)

Medial(MEC) Lateral(LEC)

Lateral Amygdaloid Nucleus(AMG)

Perirhinal Cortex (PC)

area35 ↔ area36

Unimodal and polymodal association areas

C

d slice “h”

MEC

AMI

LEC

area35

D

d slice “a”

MEC

LEC

rs

area36

1 mm

E

Electrical signals

100 µA 80 µA 60 µA 40 µA

stim. intensity

0.2 mV

0.02%

40 ms

Optical signals
Figure 2

A

PC stim. AMG stim. PC & AMG stim.

AMG

PC II/III

PC area35

MEC

DG

PC stim. LAMG stim.

B

PC (layers II/III) PC (area35)

D

(a) PC stim. (single pulse)

9.6ms 24ms 48ms 60ms 96ms 132ms 204ms 300ms 444ms

(b) AMG stim. (5 pulses at 40 Hz)

9.6ms 24ms 48ms 60ms 96ms 132ms 204ms 300ms 444ms

(c) PC (single pulse) & AMG stim. (5 pulses at 40 Hz)

5x10^{-4}

9.6ms 24ms 48ms 60ms 96ms 132ms 204ms 300ms 444ms
Figure 3

A
(a) PC stim. (single pulse)

(b) PC stim. (5 pulses at 40Hz)

(c) PC stim. (10 pulses at 40Hz)

B
(a) AMG stim. (single pulse)

(b) AMG stim. (5 pulses at 40Hz)

C
PC stim.

PC II/III

PC V/VI

LEC

AMG stim.

AMG

PC II/III

PC V/VI

LEC
Figure 4

A

(a) PC superficial layers stim. (at 40Hz)
(b) PC middle layers stim. (at 40Hz)
(c) PC deep layers stim. (single pulse)
(d) PC deep layers stim. (at 40Hz)

B

repetitive stim.
single stim.

TE
PC
EC

200 ms

2x10^4

6x10^4

0