Brief Communications

Functional Dissociation within the Entorhinal Cortex for Memory Retrieval of an Association between Temporally Discontiguous Stimuli

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Anatomical connectivity and single neuron coding suggest a segregation of information representation within lateral (LEC) and medial (MEC) portions of the entorhinal cortex, a brain region serving as the primary input/output of the hippocampus and maintaining widespread connections to many association cortices. The present study aimed to expand this idea by examining whether these two subregions differentially contribute to memory retrieval for an association between temporally discontiguous stimuli. We found that reversible inactivation of the LEC, but not the MEC, severely impaired the retrieval of the recently and remotely acquired memory in rat trace eyeblink conditioning, in which a stimulus-free interval was interposed between the conditioned and unconditioned stimulus. Conversely, inactivation of the LEC had no effect on retrieval in delay eyeblink conditioning, where two stimuli were presented without an interval. Therefore, the LEC, but not the MEC, plays a long-lasting role in the retrieval of a memory for an association between temporally discontiguous stimuli.

Introduction

Studies in humans, nonhuman primates, and rodents highlight the importance of the hippocampus and its surrounding rhinal cortices in the formation and retrieval of episodic memory (Scoville and Milner, 1957; Squire and Zola-Morgan, 1991). Among the rhinal cortices, the entorhinal cortex serves as the primary input/output structure for the hippocampus, and can be divided into caudomedial (medial entorhinal cortex, MEC) and rostro-lateral (lateral entorhinal cortex, LEC) regions. The MEC receives a greater proportion of projections from visual and visuospatial cortices, whereas the LEC receives stronger inputs from multiple association cortices arriving largely through the perirhinal cortex (Canto et al., 2008; Agster and Burwell, 2009). Consistent with this segregation in anatomical connectivity, activities of neurons in the MEC are modulated by spatial information (i.e., grid cells, Fyhn et al., 2004; Hafting et al., 2005) and are necessary for spatial memory formation and retrieval (Steffenach et al., 2005; Gaskin and White, 2010). In contrast, such spatially responsive cells are nearly absent in the LEC (Hargreaves et al., 2005).

The functional role of the LEC is less established; however, it may be involved in memory associations requiring sensory information

maintenance. The LEC receives highly processed sensory information from the perirhinal cortex (Canto et al., 2008; Agster and Burwell, 2009), and upon stimulation entorhinal neurons show persistent spiking after stimulus termination *in vitro*, lasting from tens of seconds to minutes in MEC neurons, and for an indefinite period of time in LEC neurons (Klink and Alonso, 1997; Tahvildari et al., 2007). Lesions and pharmacological manipulations to the entorhinal cortex, including medial and lateral portions, impair memory paradigms that require the maintenance of sensory information over a temporal delay (Otto and Eichenbaum, 1992; Ryou et al., 2001; Esclassan et al., 2009), however no study has investigated whether this function can be mediated specifically by the LEC. To address this point, the present study examined the effect of reversible inactivation of the LEC or MEC on memory expression in two classical eyeblink conditioning paradigms. We found that inactivation of the LEC, but not the MEC, impaired memory expression in the trace paradigm, where an auditory stimulus (conditioned stimulus, CS) and electric shock to the eyelid (unconditioned stimulus, US) were paired with an interval between them. In contrast, LEC inactivation did not impair memory expression in the delay paradigm, in which the CS coterminated with the US.

Materials and Methods

Subjects

Subjects were 24 male Long–Evans rats obtained from Charles River Laboratories at 70 days of age. Rats were housed individually in clear Plexiglas cages in a colony room with a 12 h reversed light/dark cycle (lights off at 10:00 A.M.) and given *ad libitum* access to food and water. All experimental procedures were in conformity with the guidelines and procedures outlined by the NIH Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and the Canadian Council on Animal Care, and were approved by the University of Toronto Animal Care Committee.

Figure 1. Cannula locations and spread of muscimol. *A*, A fluorescent tagged muscimol conjugate infused into the lateral (LEC, left) and medial (MEC, right) entorhinal cortex. Spread was confined to a maximal 1 mm range. A magnified view of a LEC infusion site is also provided (center). Scale bars, 600 μm. *B*, Histological reconstructions of cannula locations in the LEC (left) and MEC (right) of the animals included in the analyses. In 18 animals (1 d retention, Recent, $n = 8$, red circles; 1 month retention, Remote, $n = 10$, black circles), the cannulae were bilaterally positioned in the LEC, and of those 18 animals, 12 also had bilateral cannulae in the MEC (Recent, $n = 6$; Remote, $n = 6$). Images were adapted from Paxinos and Watson, Copyright Elsevier (1998) with permission.

Design

All rats were trained in a trace eyeblink conditioning paradigm, followed by a 6 d period of retention testing. Before conditioning, rats were randomly assigned to two groups; the Recent group received retention sessions 1 d after conditioning, and the Remote group was tested on retention 1 month after conditioning. Following the last retention session, all rats underwent one acquisition and two retention sessions in a delay eyeblink conditioning paradigm.

Surgery

Rats received one of two surgery protocols based on group assignment: (1) rats in the Recent group were implanted with electrodes for electromyogram (EMG) and periorbital shock, and guide cannulae for drug infusion, before acquisition sessions ($n = 12$); (2) rats in the Remote group received electrode implantation only, then electrode replacement and guide cannulae implantation 1 week before retention testing. Surgeries followed similar protocols as reported previously (Takehara et al., 2003). Briefly, under anesthesia with isoflurane (1–1.5% by volume in oxygen at a flow rate of 1.5 L/min; Holocarbon Laboratories) rats had four Teflon-coated stainless-steel wires implanted subcutaneously in the *obicularis occuli* muscle of the upper left eyelid for EMG recording and periorbital shock delivery, and another wire implanted into the neck for reference; all wires were attached to a small connector cap. Small burr holes were drilled bilat-

erally on the skull, and guide cannulae (Plastics One) were lowered at the following coordinates: For the LEC: -6.45 anteroposterior (AP), ±6.65 mediolateral (ML), and -8.5 mm dorsoventral (DV) to bregma; for the MEC: -8.88 AP, ±4.7 ML, and -5.3 mm DV. All coordinates were adjusted from the rat atlas of Paxinos and Watson (1998) based on histology in our pilot study. The cannulae and connector cap were then secured to the skull with dental acrylic and stainless-steel screws, and cannulae were capped with dummy stylets.

Intracerebral drug infusion

Before each retention session, rats were placed in plastic restrainers (Stoelting) and received bilateral infusions in the LEC, MEC, or both, at a speed of 0.5 μl per min of either muscimol [Muscimol HBr, Sigma, 0.5 μg in 0.5 μl of artificial CSF (aCSF)] or 0.5 μl of aCSF using a microinfusion pump (Harvard Apparatus). On each of six retention days, individual rats received one of six possible infusions of either muscimol (Mus) or aCSF in the MEC, LEC, or both: [(1) Mus-LEC; (2) Mus-MEC; (3) Mus-LEC and MEC; (4) aCSF-LEC; (5) aCSF-MEC; (6) aCSF-LEC and MEC] at least 10 min before the start of the session. Infusion schedules were generated randomly, with all rats receiving each infusion only once. The experimenter was blind to the infusion schedules.
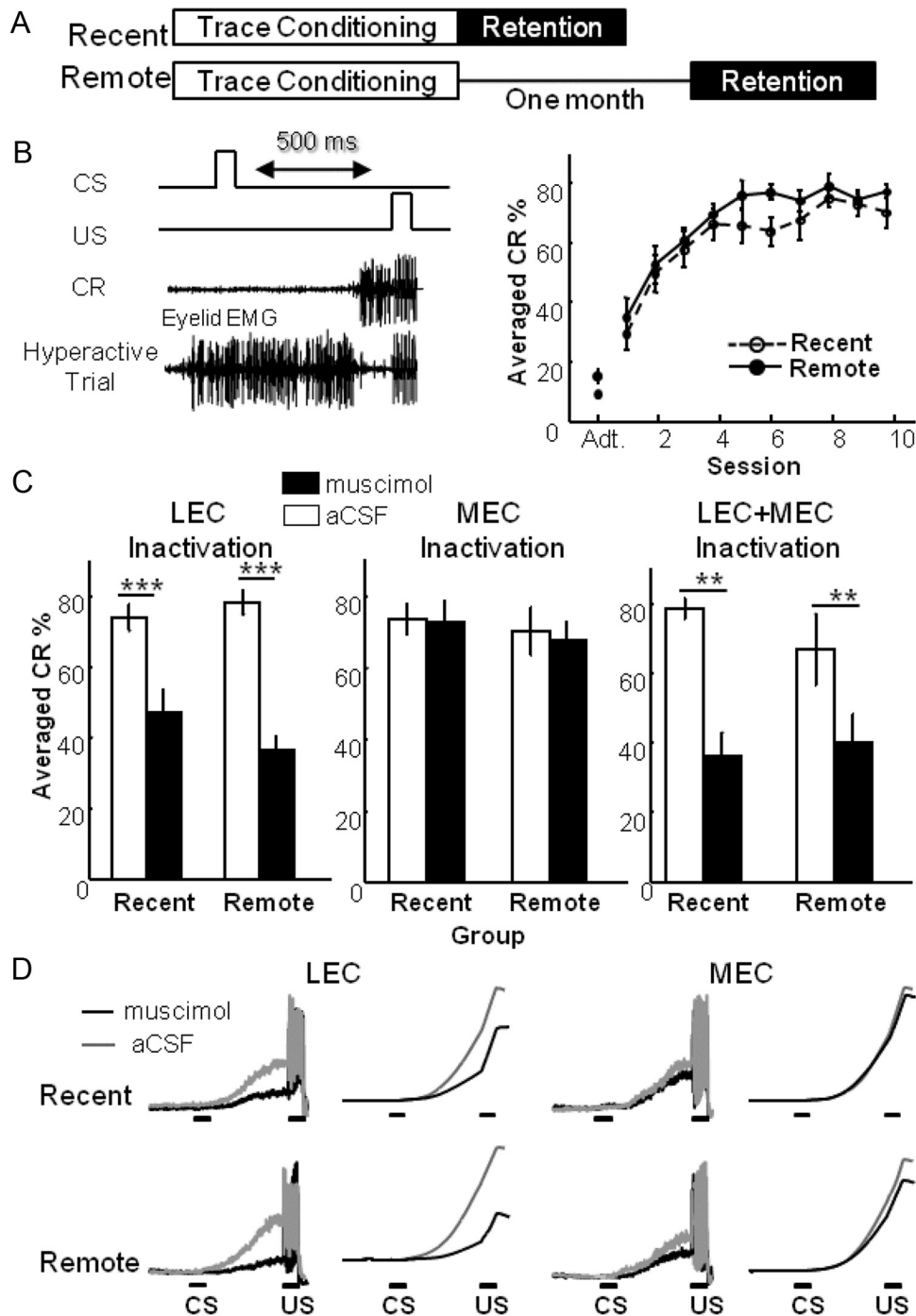
Figure 2. Design, acquisition, and retention in trace conditioning. ***A***, Rats were trained in the trace paradigm and tested 1 d (Recent group) or 1 month (Remote group) after training. ***B***, Conditioning sessions consisted of a conditioned stimulus (CS, 100 ms tone) paired with an unconditioned stimulus (US, 100 ms periorbital shock) separated by a 500 ms stimulus-free interval. Animals' eyeblink responses were monitored by recording electromyogram (EMG) in *obicularis occuli* (top). Approximately 15 of 100 trials were discarded because of hyperactivity before the CS presentation (bottom). Before conditioning, two adaptation sessions (Adt.) recorded spontaneous eyeblinks in the absence of CS or US. The Recent and Remote groups acquired the conditioned response (CR), but were not different from one another. ***C***, Muscimol infusion into the lateral entorhinal cortex (LEC, black bar) severely impaired CR expression compared with aCSF infusion (white bar) at recent ($n = 8$) and remote ($n = 10$) time-points (left). No effect was observed after muscimol infusion into the MEC (center) at either time-point (Recent, $n = 6$; Remote, $n = 6$). Combined muscimol infusion into the LEC and MEC impaired CR expression compared with aCSF infusion at recent ($n = 6$) and remote ($n = 6$) test times (right). **$p < 0.01$, ***$p < 0.001$. ***D***, The increase in averaged amplitude of EMG and the integrated area of the CR following CS onset during sessions with muscimol infusion (black line) into the LEC (left) was smaller than that during sessions with aCSF infusion into either region (gray line) or muscimol infusion into the MEC (right) at both recent (top) and remote (bottom) time points.

Eyeblink conditioning

Conditioning apparatus. Rats were placed in clear cylindrical Plexiglas containers and into one of four sound- and light-attenuating conditioning chambers (Med Associates). Lightweight cables were attached to the pins of the connector secured to the animals head for recording EMG

activity and shock delivery. A speaker on the chamber ceiling presented the CS, a 100 ms tone (2.5 kHz, 85 dB). The US was a 100 ms periorbital shock (100 Hz square pulses). In trace eyeblink conditioning, a stimulus-free trace interval (500 ms) was interposed between CS offset and US onset. In delay eyeblink conditioning, the CS was extended to 350 ms

and coterminated with the US. The US intensity was initiated at 0.3 mA and carefully calibrated throughout sessions to ensure the minimal current was provided to elicit an eyeblink/head-turn response, which was monitored via web-cameras mounted inside the chambers. The presentations of the CS and US were controlled by a microcomputer (BasicX, Netmedia).

Behavioral procedure. Conditioning began no less than 3 d after electrode surgery (7 d after the implantation of electrodes and cannulae). The acquisition sessions took place once per day for 12 d during the dark phase of the reversed light/dark cycle between 1:00 and 5:00 P.M. Days 1–2 allowed rats to acclimate to the procedures in the absence of the CS or US while the frequency of spontaneous eyeblinks was recorded. Presentations of the CS and US began on day 3. Each session consisted of a 10 min acclimation period followed by 100 trials of CS–US pairings over a 50 min span, during which EMG activity was continuously recorded from the *obicularis occuli* muscle with a RZ-5 recording system (Tucker-Davis Technologies). EMG activity was filtered between 0.3 and 3 kHz and digitized at 6 kHz. Trials were grouped into 10 trial blocks, with nine paired CS–US presentations followed by one CS only trial. Intervals between trials were pseudorandomized between 20 and 40 s with a mean of 30 s. Retention sessions followed exactly the same protocol as acquisition sessions.

EMG analysis

Similar to a previous study (Takehara et al., 2003), amplitude of EMG activity (EMG amplitude) was calculated by subtracting the minimum from maximum EMG signals in a 1 ms window. A threshold was determined by calculating the mean + SD of EMG amplitude during the 300 ms window before CS onset over 100 trials (including both trials with a CS–US pairing and trials with CS alone presentation). For each trial, EMG amplitudes above this defined threshold were averaged during the 300 ms period before CS onset (prevalue) and the 200 ms period before US onset [conditioned response (CR) value]. Trials were discarded for hyperactivity when the prevalue exceeded 30% of the threshold (~15 of 100 trials). If the CR value exceeded 5× the prevalue, the trial was defined as containing a CR. A CR percentage was calculated for each animal for each session as the ratio of CR trials to the total number of valid trials. For visual confirmation, we calculated the averaged EMG amplitude and integrated area of the CR for the 900 ms period beginning 300 ms before CS onset until US onset across rats. The integrative area of the CR constitutes the cumulative summation of the averaged EMG signals minus the prevalue for all valid trials.

Histology

Upon completion of all behavioral tests, rats received intraperitoneal injections of an excess amount of sodium pentobarbital (150 mg/kg body weight, Euthansol, Merck) and perfused intracardially with 0.9% saline, followed by 10% phosphate-buffered formalin. After cryoprotection, brain tissue was sectioned horizontally at 50 μm thickness, mounted on slides, and stained with cresyl violet. Cannula positioning was then identified using a light microscope.

Muscimol spread analysis

To confirm the spread of muscimol around the injected site, a fluorescent tagged muscimol (muscimol BODIPY TMR-X conjugate, Invitrogen) was infused bilaterally into the LEC and MEC of a few selected rats. Forty minutes after infusion, the rats were killed and perfused. Tissue was sectioned in a coronal plane at 50 μm thickness, mounted on slides and imaged with a fluorescent microscope (Zeiss) with a DsRed filter cube. Images were captured with a Retiga EXi camera (Q-Imaging), which was coupled to the microscope, and imaging software (Volocity, PerkinElmer).

Statistical analyses

Data are expressed as the mean ± SEM. To determine statistical significance, we used two-way repeated-measures ANOVA with sessions or treatments as a within-subjects factor and retention intervals as a between-subjects factor. When appropriate, paired-samples *t* tests were used. Criterion for significance was set at $p < 0.05$.
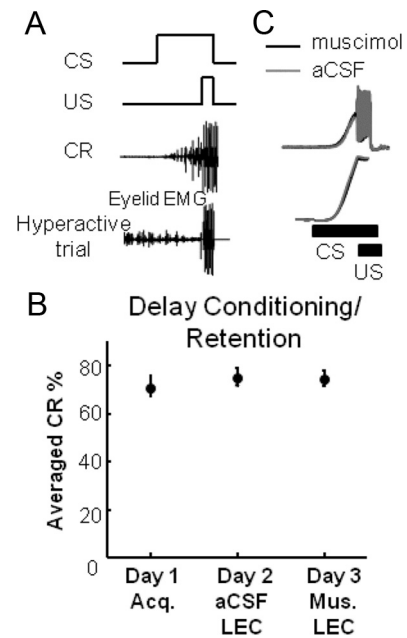
Figure 3. Design, acquisition, and retention in delay conditioning. *A*, Following retention sessions in trace conditioning, the rats received one acquisition session (Acq.) and two retention sessions in a delay paradigm where the conditioned stimulus (CS, 350 ms tone) coterminated with the unconditioned stimulus (US, 100 ms periorbital shock). *B*, In the session with muscimol (Mus.) infusions into the lateral entorhinal cortex (LEC), rats showed a comparable percentage of the conditioned response (CR) to the session with aCSF infusion (*n* = 14). *C*, The temporal pattern of averaged EMG amplitude or integrated area of the CR following CS onset did not differ between sessions with Muscimol infusion (black line) and sessions with aCSF infusion (gray line).

Results

Histology and muscimol spread analysis

Histological results confirmed that most cannulae were implanted in their target positions (Fig. 1). A total of 18 rats had cannula in the LEC in both hemispheres. Of these 18 rats, a total of 12 rats also had cannulae bilaterally within the MEC. All animals in which both cannulae were not correctly positioned in the LEC were removed from further analysis.

Upon completion of behavioral trainings, four rats were injected with a conjugate of muscimol and the BODIPY TMR-X fluorophore. The fluorescent signals were detected specifically in the regions of interest, and spread was confined to a maximal 1 mm range (Fig. 1*A*), consistent with a previous study (Allen et al., 2008).

Effects of LEC and MEC inactivation on the memory retrieval of an association between temporally discontiguous stimuli

Either 1 d (Recent group) or 1 month (Remote group) after the 10th acquisition session in trace eyeblink conditioning, memory retention was tested with infusion of either muscimol or aCSF into the LEC, MEC, or both (Fig. 2*A*). All rats acquired the conditioned response over the course of acquisition, with no differences between groups (Fig. 2*B*; two-way repeated-measures ANOVA; session, $F_{(11,176)} = 83.876$, $p < 0.0001$; group, $F_{(1,16)} = 2.139$, n.s.; interaction, $F_{(11,176)} = 0.525$, n.s.).

During retention sessions 1 d after the last acquisition session (Recent group), infusion of muscimol into the LEC significantly impaired the expression of CRs compared with aCSF infusion. A similar impairment was observed in the Remote group, when muscimol was infused into the LEC before testing retention of the memory acquired 1 month earlier (Fig. 2*C*; two-way repeated-measures ANOVA; infusion, $F_{(1,16)} = 80.512$, $p < 0.001$; group,

$F_{(1,16)} = 0.414$, n.s.; interaction, $F_{(1,16)} = 3.624$, n.s.). Conversely, there were no effects of muscimol infusion into the MEC on the expression of CRs either 1 d or 1 month after training (infusion, $F_{(1,10)} = 0.85$, n.s.; group, $F_{(1,10)} = 2.336$, *n.s.*; interaction, $F_{(1,16)} = 2.921$. n.s.). In addition, inactivation of both the LEC and MEC produced no further impairment on the expression of CRs than inactivation of the LEC alone (infusion into the LEC only or both the LEC and MEC, $F_{(1,10)} = 0.198$, n.s.; group, $F_{(1,10)} = 0.718$, n.s.; interaction, $F_{(1,10)} = 1.809$, n.s.).

Visual inspection of EMG activity confirmed a reduction of CR expression following muscimol infusion into the LEC (Fig. 2*D*). In all sessions with aCSF infusion or muscimol infusion into the MEC, the averaged EMG amplitude and integrated area of the CR gradually increased from CS offset to US onset. This increase was much smaller in sessions with muscimol infusion into the LEC. Finally, infusion had no effect on trial selection, as the average number of trials removed each session (because of hyperactivity) did not differ between the LEC muscimol infusion (mean = 15.1 ± 1.35) and the LEC aCSF infusion sessions (mean = 15.22 ± 1.44; $t_{(17)} = -.113$, n.s.).

Effects of LEC inactivation on the memory retrieval of an association between temporally contiguous stimuli

To confirm that the observed effects of LEC inactivation were specific to the association between discontiguous stimuli, following trace eyeblink retention sessions, all animals were trained and tested in delay eyeblink conditioning, where the CS coterminated with the US (Fig. 3*A*). Delay conditioning consisted of 1 d of training, followed by 2 d of retention where aCSF or muscimol were infused into the LEC. In the session with muscimol infusion, the rats exhibited a comparable percentage of CRs to the session with aCSF infusion (Fig. 3*B*, paired-samples *t* test, $t_{(13)} = 0.376$, n.s.). The averaged EMG amplitude and integrated area of the CR across trials also did not differ between the session with aCSF infusion and the session with muscimol infusion (Fig. 3*C*). These results suggest that the effect of LEC inactivation was specific to memory expression in trace eyeblink conditioning, in which an association must be formed between discontiguous stimuli.

Discussion

We revealed that inactivation of the LEC, but not the MEC, impaired memory expression in trace eyeblink conditioning, in which rats were required to form an association between temporally discontiguous stimuli. The observed impairment could not be attributed to impairments in sensory processing or motor coordination, because LEC inactivation had no effect on memory expression in delay eyeblink conditioning involving the same stimuli and behavioral monitors, but no imposed interval between the stimuli.

Consistent with previous research (Otto and Eichenbaum, 1992; Ryou et al., 2001; Esclassan et al., 2009), the present results reveal an important contribution of the entorhinal cortex to mnemonic processes during paradigms requiring the maintenance of sensory information over a temporal delay. Theoretical models (Hasselmo et al., 2000; Hasselmo and Stern, 2006) have related this function of the entorhinal cortex to its self-sustaining spike activity observed *in vitro* (Egorov et al., 2002; Tahvildari et al., 2007) and persistent activity during a delay period following stimulus presentation *in vivo* (Suzuki et al., 1997; Young et al., 1997). Although persistent activity can be observed in both the LEC and MEC (Egorov et al., 2002; Tahvildari et al., 2007), the LEC is more strongly connected with the perirhinal cortex, whose projections mediate highly processed sensory information (Fur-

tak et al., 2007), and with the prelimbic medial prefrontal cortex (Hoover and Vertes, 2007; Agster and Burwell, 2009), which plays a critical role in memory retrieval in trace eyeblink conditioning (Takehara-Nishiuchi et al., 2005; Oswald et al., 2010). Furthermore, these two regions show differential selectivity for spatial versus nonspatial information (Hargreaves et al., 2005). These differences in anatomical connectivity between the LEC and MEC may explain the observed functional dissociation in the trace paradigm, whereby the LEC may function to maintain CS information during the trace interval by sustained neuronal activity, enabling hippocampal or prefrontal networks to recover patterns of neuronal activities selective for the previously acquired association (McEchron and Disterhoft, 1997; Takehara-Nishiuchi and McNaughton, 2008). Targeted manipulations to self-sustaining entorhinal activity using a muscarinic or metabotropic glutamate receptor antagonist (Klink and Alonso, 1997; Yoshida et al., 2008; Esclassan et al., 2009) could test this possibility.

The hippocampus plays a critical role in forming an association between two stimuli with a temporal delay (Moyer et al., 1990; McEchron et al., 1998; Chowdhury et al., 2005), however, its involvement in memory expression is time-limited (Kim et al., 1995; Takehara et al., 2003; Quinn et al., 2008). In contrast, we found a long-lasting role for the LEC in memory retrieval, as LEC inactivation impaired expression of both recent (1-d-old) and remote (1-month-old) memories in trace eyeblink conditioning. This is consistent with findings of long-lasting retrograde memory impairments when large hippocampal lesions include the rhinal cortices in humans (Bayley et al., 2006) and primates (Suzuki et al., 1993), and with specific lesions to subregions of the rhinal cortices in rodents (Cho and Kesner, 1996; Burwell et al., 2004). Together, these findings suggest that the rhinal cortices play a long-lasting role in memory storage or retrieval. In a spatial memory task, however, entorhinal involvement in memory expression has been shown to decrease 6 weeks after training (Cho and Kesner, 1996), suggesting that the rhinal cortices may be involved in a more extended consolidation process than the hippocampus and may eventually disengage from the network supporting memory storage and expression. Future electrophysiological studies will help discriminate these two possibilities by examining activity profiles of entorhinal neurons in the context of interaction with other neocortical regions that are presumed to store permanent memory.

In conclusion, the present study demonstrates a long-lasting role for the LEC in memory retrieval for an association between temporally discontiguous stimuli, providing further support for a functional dissociation between the LEC and MEC.

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