

## Brief Communication

# Plasticity in the Entorhinal Cortex Suppresses Memory for Contextual Fear

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Several studies have delineated a role for the hippocampus in fear conditioning. However, in this task, the role of the entorhinal cortex (EC), the main input–output structure for the hippocampus, is uncertain. The extracellular signal-regulated kinase (ERK) cascade has been shown to be a molecular correlate for long-term memory, and its activity is required for various types of memory storage, including fear memory. In this study, we show that ERK activity in the EC increased 90 min after fear conditioning. Post-training intra-EC infusion of ERK cascade inhibitors (PD098059, UO126) at 40 min, but not at 10 min, resulted in increased freezing to the context, but not to the tone, during a 48 hr retention test. Interestingly, both PD098059- and UO126-infused animals also demonstrated anticipatory freezing in the context, freezing maximally at the time the shock was given during training. This anticipatory behavior was also seen in naive animals receiving additional training. Together, these results suggest that ERK-mediated plasticity in the EC normally suppresses context-specific fear memory, especially the temporal nature of the freezing response, and that blocking this plasticity mimics the effects of additional training.

**Key words:** anticipation; ERK; fear conditioning; amygdala; hippocampus; delay conditioning

## Introduction

Cued fear conditioning is a type of Pavlovian conditioning in which a tone is paired with a foot shock in a distinctive context, eliciting a fear response to both the context and the tone. Although the amygdala is required for both cued (tone) and contextual fear, the hippocampus is only involved in the acquisition and storage of contextual representations (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Maren et al., 1997; Anagnostaras et al., 1999). The hippocampus receives major input from, and sends output through, the entorhinal cortex (EC) (Burwell et al., 1995), suggesting that the EC may also be involved in context-specific fear memory. However, lesion studies report conflicting results, with electrolytic lesions of the EC resulting in deficits (Maren and Fanselow, 1995) and neurotoxic or aspiration lesions having no effect (Phillips and LeDoux, 1995; Bannerman et al., 2001). Furthermore, all lesions were performed before training, making it difficult to disassociate effects on memory retention from effects on acquisition.

Post-training treatments are highly effective for examining memory storage without altering memory acquisition (McGaugh, 1966; Schafe and LeDoux, 2000). PD098059 and UO126, cell-permeable inhibitors specific for the extracellular signal-regulated kinase (ERK) cascade (Alessi et al., 1995; Favata et al.,

1998; Davies et al., 2000) have been used extensively to demonstrate a role for ERK-mediated plasticity in long-term memory storage (Berman et al., 1998; Blum et al., 1999; Walz et al., 1999; Kanterewicz et al., 2000; Schafe et al., 2000; Crow et al., 2001; Hebert and Dash, 2002; Runyan et al., 2004). Furthermore, blockade of the ERK cascade does not affect normal neuronal activity (English and Sweatt, 1997), short-term memory, or acquisition (Blum et al., 1999). Targeted blockade of the ERK cascade in the hippocampus or amygdala results in long-term memory deficits in trace (Runyan et al., 2004) and delay (Schafe et al., 2000) fear conditioning, respectively. These and other studies indicate that post-training reversible blockade of the ERK cascade within a brain structure can be used to assess its role, specifically in memory storage, without interfering with normal neuronal function. In this study, we examine the role of the EC in long-term memory storage of fear conditioning by combining targeted blockade of plasticity within the EC using ERK cascade inhibitors, with a post-training treatment protocol.

## Materials and Methods

**Subjects.** Adult male Long–Evans rats (220–250 gm) from Charles River Laboratories (Wilmington, MA) were maintained on a 12 hr light/dark cycle with *ad libitum* access to food and water. Protocols involving animals were in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Welfare Committee.

Rats were anesthetized with isoflurane and implanted with bilateral guide cannulas aimed 1.5 mm above the EC (anteroposterior,  $-6.7$ ; lateral,  $\pm 5.5$ ; ventral, 6.0) (Paxinos and Watson, 1997). Animals recovered for 10 d before behavioral testing.

**Drug preparation and infusion.** PD098059 (Biomol, Plymouth Meet-

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ing, PA) and UO126 (Calbiochem, La Jolla, CA), initially dissolved in DMSO, were diluted in sterile saline for the infusions. Freely moving animals were bilaterally infused with 1  $\mu$ l per side of drug (0.2  $\mu$ g of PD098059 in 25% DMSO; 1  $\mu$ g of UO126 in 35% DMSO) or vehicle into the EC using a dual-syringe infusion pump (Stoelting, Wood Dale, IL) at a rate of 0.25  $\mu$ l/min. Infusion cannulas were left in for an additional 2 min to allow for diffusion. The infusion cannulas extended 1.5 mm beyond the guide cannulas for a final depth of 7.5 mm.

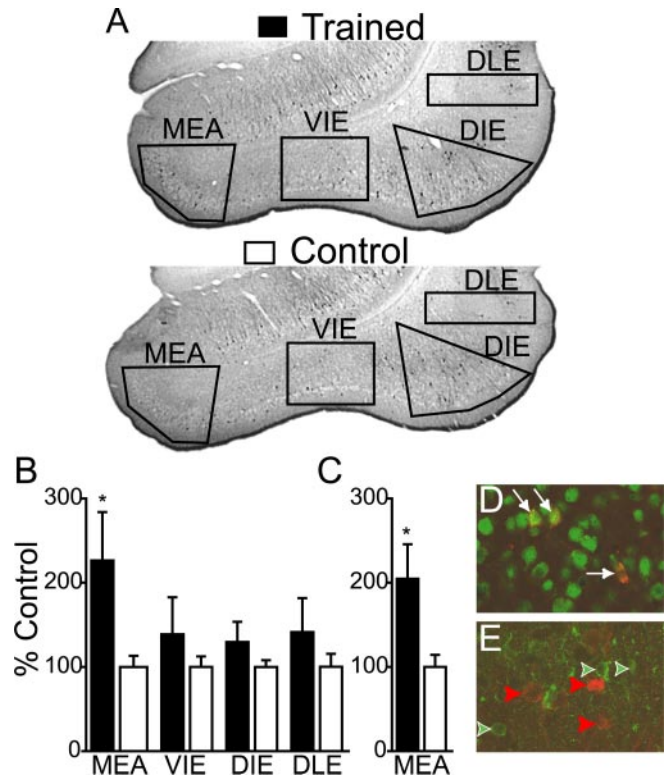
**Immunohistochemistry.** Dual phosphorylation of ERK is an indication of its activation (Payne et al., 1991). To detect ERK activation, immunohistochemistry was performed on 40  $\mu$ m free-floating slices using anti-dual-phospho-ERK1/2 (1  $\mu$ g/ml; Cell Signaling Technologies, Beverly, MA) and visualized using ABC and DAB kits (Vector Laboratories, Burlingame, CA) as described previously (Hebert and Dash, 2002). For double staining, anti-dual-phospho-ERK1/2 and either anti-GAD67 (2  $\mu$ g/ml; Chemicon, Temecula, CA) or anti-neuron-specific nuclear protein (NeuN) antibodies (0.5  $\mu$ g/ml; Chemicon) were visualized using species-specific Alexa-conjugated secondary antibodies. To check for double-stained cells, all of the phospho-ERK-positive cells were marked, the filter was changed, and all of the GAD67-positive cells were marked. Any double labeling was confirmed using a confocal microscope.

**Cell counts.** All of the cell counts were performed by an experimenter blind to the treatment groups. Ninety minutes after training (trained group) or an equivalent exposure to the tone and context but with no shock (control group), animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with fixative (4% paraformaldehyde and 15% picric acid in 1 $\times$  PBS). Brains were removed, cut along the midline, postfixed for 24 hr, cryoprotected in 30% sucrose, and coronal sections were cut at 40  $\mu$ m using a cryostat. Either the left or right hemisphere (counterbalanced between animals) was processed for immunohistochemistry. Cytoarchitectonic subfields of the EC were identified using cresyl violet-stained adjacent sections and the nomenclature of Insausti et al. (1997). Counts of phospho-ERK-positive profiles within each of the defined areas were taken from four consecutive sections per animal at 10 $\times$  magnification. The counts for each area were averaged to produce a mean and were converted to percentage of matched controls. Any significant increases seen in a subfield of the EC were confirmed by recounting that area using a nonbiased stereological technique, the optical disector, which was described previously (Coggeshall, 1992).

**Behavioral training.** All behavior was performed by an experimenter blind to the treatment groups. Each rat was placed in the training chamber and a 30 sec tone was presented after 2 min of exploration. A 1.0 mA foot shock was given during the last 2 sec of tone presentation. After two training trials and a 1 min rest period, animals were returned to their home cages. Memory for the context and for the tone was tested 48 hr later by measuring the percentage of freezing. Freezing was defined as the complete lack of movement except that required for breathing. To test for contextual memory, animals were placed back in the training chamber for 3 min, and freezing behavior was measured at 3 sec intervals. To test for cued memory, the same animals were then placed in a novel context for 2 min, followed by a 3 min tone presentation. Freezing behavior was monitored and recorded both before (novel context) and during the tone presentation (tone).

**Verification of cannulas placement.** After behavioral testing, animals were killed and bilaterally infused with 1.5  $\mu$ l of Coomassie blue dye. The brains were removed, cut into 4-mm-thick slabs, postfixed in 4% paraformaldehyde and 15% picric acid for 24–48 hr, cryoprotected in 30% sucrose, and cut at 40  $\mu$ m using a cryostat. Sections including the infusion sites were stained with cresyl violet. Animals with infusion sites within the EC were kept for additional analysis.

**Statistical analysis.** Statistical significance for differences in total percentage of freezing between two groups was determined using Mann–Whitney rank sum tests and between multiple groups using ANOVA. Statistical significance for differences in cell count percentages and in the binned data between drug- and vehicle-retention test freezing curves was determined using two-way repeated-measures ANOVA followed by *post*



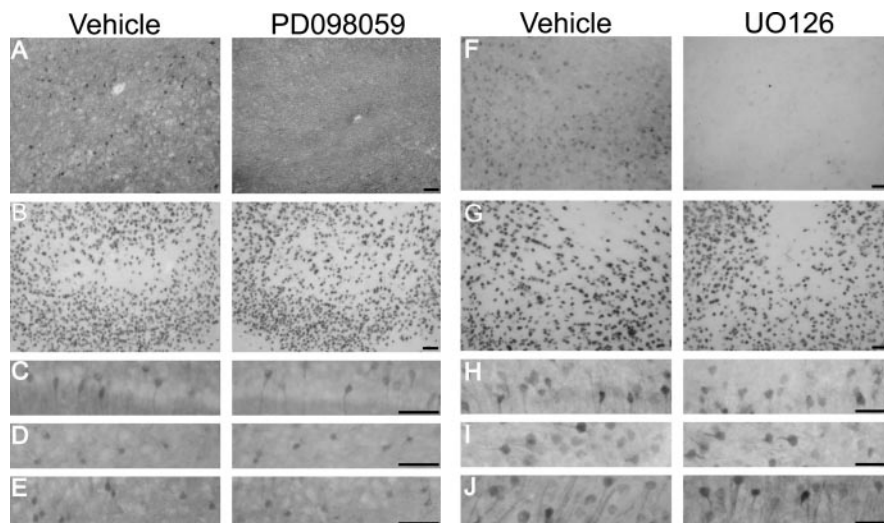
**Figure 1.** Fear conditioning increases ERK phosphorylation in the entorhinal cortex 90 min after training. *A*, Representative photomicrographs showing the areas for cell counting after training ( $n = 6$ ) or tone–context (control) exposure ( $n = 7$ ). *B*, *C*, Summary of phospho-ERK-positive profile counts at 10 $\times$  (*B*) and cell counts in MEA using unbiased stereology (*C*). *D*, *E*, Representative confocal photomicrographs of phospho-ERK (red)–NeuN (*D*) or GAD67 (*E*) double staining in the EC. Arrows mark double staining (*D*); arrowheads mark nondouble-stained cells; red arrowheads mark phospho-ERK-positive cells, and green arrowheads mark GAD67-positive cells (*E*). VIE, Ventral intermediate entorhinal; DIE, dorsal intermediate entorhinal; DLE, dorsal lateral entorhinal. Data indicate mean  $\pm$  SEM; \* $p < 0.05$ .

*hoc* analysis when appropriate. Data were presented as mean  $\pm$  SEM and were considered significant at  $p < 0.05$ .

## Results

### Fear conditioning increases ERK phosphorylation in the entorhinal cortex

Previous studies have shown an increase in ERK phosphorylation in the hippocampus  $\sim$ 1 hr after training in both delay and trace fear conditioning (Atkins et al., 1998; Runyan et al., 2004). To examine ERK phosphorylation in the EC after delay fear conditioning, tissue from trained and control animals was processed for immunohistochemistry. Figure 1*A* shows representative photomicrographs of trained and control coronal sections of the EC, with the counting areas marked. Fear conditioning resulted in a significant increase in phospho-ERK staining in the EC 90 min after training (two-way ANOVA; trained vs control,  $p < 0.05$ ). *Post hoc* analysis demonstrated a significant increase in the number of phospho-ERK-positive profiles in the medial entorhinal area (MEA) ( $p < 0.05$ ) but not in the ventral intermediate, dorsal intermediate, or dorsal lateral entorhinal area (LEA) (Fig. 1*B*). The increase in the MEA was confirmed using unbiased stereology ( $p < 0.05$ ) (Fig. 1*C*). Phospho-ERK is expressed in both excitatory and inhibitory neurons. To determine which cell types showed increased phospho-ERK immunoreactivity, slices from trained and control animals were double stained either with phospho-ERK and NeuN (a neuronal-specific marker) or phospho-ERK and GAD67 (a GABAergic marker). Phospho-



**Figure 2.** Intraentorhinal infusion of PD098059 or UO126 decreases phospho-ERK immunoreactivity (A, F) but not NeuN immunoreactivity (B, G) in the entorhinal cortex and does not affect phospho-ERK immunoreactivity in the dorsal hippocampus (C, H), ventral subiculum (D, I), or ventral hippocampus (E, J) compared with the vehicle-infused contralateral side. Scale bars, 50  $\mu$ m.

ERK-positive cells colocalized with NeuN-positive cells (Fig. 1D) but not with GAD67-positive cells (Fig. 1E).

#### Blockade of ERK phosphorylation is localized to the entorhinal cortex after intra-EC infusion of PD098059 or UO126

To confirm that phospho-ERK blockade was localized to the EC, basal phospho-ERK immunoreactivity was examined after PD098059 or UO126 infusion into one side and vehicle infusion into the contralateral side of the same animal, as described previously (Hebert and Dash, 2002). Phospho-ERK immunoreactivity was reduced in both the MEA and the LEA after PD098059 (Fig. 2A) or UO126 (Fig. 2F) infusion compared with the vehicle-infused contralateral side. This reduction was not attributable to a loss of cells or immunoreactivity, because there was no difference in NeuN immunoreactivity in the EC (Fig. 2B, G). Intra-EC PD098059 or UO126 infusion did not affect surrounding structures. Phospho-ERK immunoreactivity in the dorsal hippocampus (Fig. 2C, H), ventral subiculum (Fig. 2D, I), and ventral hippocampus (Fig. 2E, J) was unaffected.

Intrahippocampal infusion of PD098059 results in a transient inhibition of phospho-ERK that dissipates by 1 hr after infusion (Blum et al., 1999), and phospho-ERK immunoreactivity in the EC is decreased 40 min after intra-EC PD098059 infusion (Hebert and Dash, 2002). Based on these data, it is anticipated that a 40 min post-training PD098059 infusion would result in the inhibition of phospho-ERK up to  $\sim$ 100 min after training, up to and including the time in which an increase in phospho-ERK activation was detected (90 min). However, PD098059 infused 10 min after training should dissipate before the increase in ERK activation.

#### Blockade of ERK-mediated plasticity in the EC 40 min after training enhances long-term memory for the context

To determine whether the observed increase in ERK activity is behaviorally relevant, PD098059 was infused into the EC 40 min after training. Nonredundant infusion sites for all of the behavioral experiments are shown in Figure 3A. The majority of infusion sites fell within the LEA; however, the guide cannulas were

aimed with a slight angle toward the midline, resulting in preferential drug diffusion toward the MEA. Before treatment, both groups demonstrated equivalent acquisition (PD098059,  $86.7 \pm 6.7\%$  freezing; vehicle,  $77.9 \pm 7.5\%$  freezing; NS). Animals infused with PD098059 demonstrated significantly higher total freezing to the training context during the long-term retention test compared with vehicle-infused animals (PD098059,  $62.3 \pm 12.8\%$ ; vehicle,  $24.8 \pm 8.8\%$ ;  $p < 0.05$ ).

In addition, analysis of the freezing behavior, broken down as percentage of freezing for successive 30 sec time bins, demonstrated a significant difference between the two groups ( $p < 0.05$ ), with an anticipatory freezing effect in the PD098059-infused group ( $p < 0.05$ ) but not in the vehicle-infused group (Fig. 3B). Anticipatory freezing is defined as low levels of freezing during the initial portion of the context-retention test (similar to controls), with a significant increase in freezing

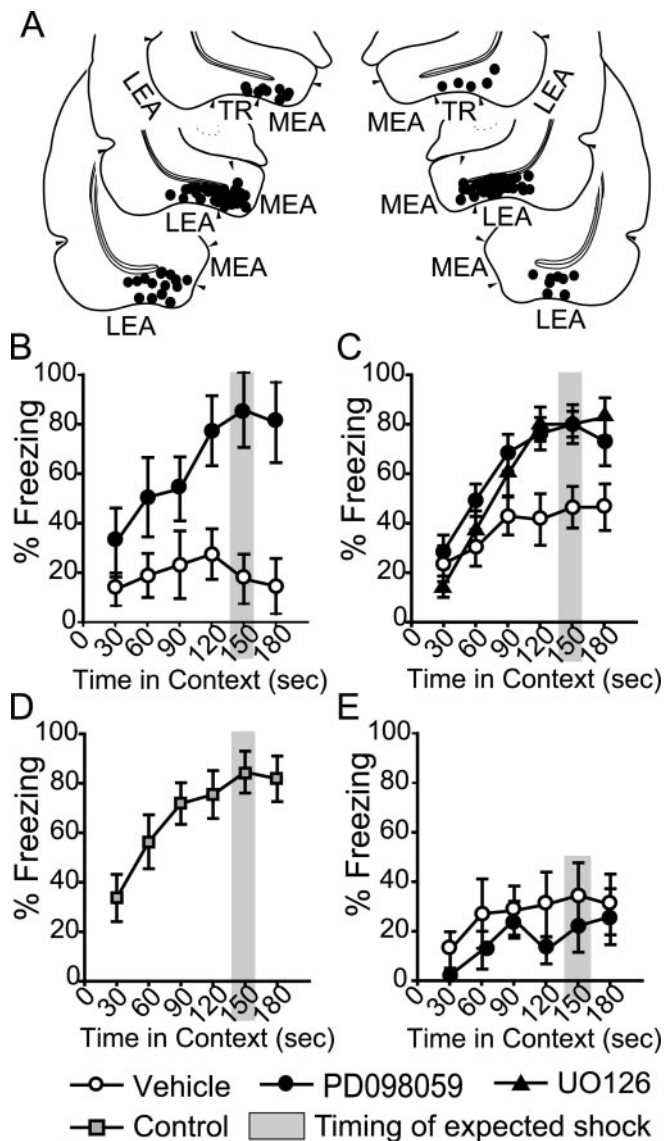
over time and maximal freezing around the time when the shock was presented during training. There were no differences between the groups in total percentage of freezing to the tone (PD098059,  $57.9 \pm 10.7\%$ ; vehicle,  $45.2 \pm 7.0\%$ ; NS) or to the novel context (PD098059,  $5.8 \pm 3.1\%$ ; vehicle,  $2.1 \pm 1.1\%$ ; NS).

The role of ERK activity was further examined by repeating the PD098059 experiment, as well as with an additional experiment using a structurally different mitogen-activated protein kinase kinase inhibitor, UO126. Both the PD098059 and the UO126 infusions into the EC resulted in an increase in total freezing to the context during the long-term retention test (PD098059,  $61.3 \pm 4.3\%$ ; UO126,  $59.1 \pm 5.4\%$ ; vehicle,  $38.3 \pm 6.4\%$ ;  $p < 0.05$ ) as well as in anticipatory freezing ( $p < 0.05$ ) (Fig. 3C). All of the groups learned to the same extent (PD098059,  $69.4 \pm 6.1\%$  freezing; UO126,  $72.1 \pm 5.5\%$  freezing; vehicle,  $60.0 \pm 7.1\%$  freezing; NS), and there were no differences in total freezing to the novel context (PD098059,  $7.8 \pm 2.6\%$ ; UO126,  $6.4 \pm 2.6\%$ ; vehicle,  $4.4 \pm 1.3\%$ ; NS) or to the tone (PD098059,  $77.8 \pm 5.1\%$ ; UO126,  $76.2 \pm 10.4\%$ ; vehicle,  $62.1 \pm 7.8\%$ ; NS). Although there were subtle differences in the freezing behavior of animals compared between the different drug groups and between the different vehicle groups, these differences were not statistically significant.

If ERK-mediated plasticity in the EC is acting to suppress contextual freezing and anticipation, additional training may overcome this inhibition. Consistent with this, naive animals given a total of six training trials over 2 d demonstrated significant anticipatory freezing ( $p < 0.05$ ) and levels of freezing similar to EC-drug-infused animals during the context-retention test (Fig. 3D).

To further specify the time course for the training-related versus generic requirement of ERK activity in the EC, intra-EC PD098059 infusion was performed 10 min after fear conditioning. Animals learned to the same degree before PD098059 infusion (PD098059,  $72.5 \pm 7.5\%$  freezing; vehicle,  $78.7 \pm 3.9\%$  freezing; NS). Ten minutes after training, intra-EC PD098059 infusion did not affect total levels of freezing to the context (PD098059,  $16.8 \pm 5.7\%$ ; vehicle,  $27.4 \pm 10.3\%$ ; NS), to the tone (PD098059,  $50.4 \pm 11.1\%$ ; vehicle,  $56.2 \pm 13.5\%$ ; NS), or to the



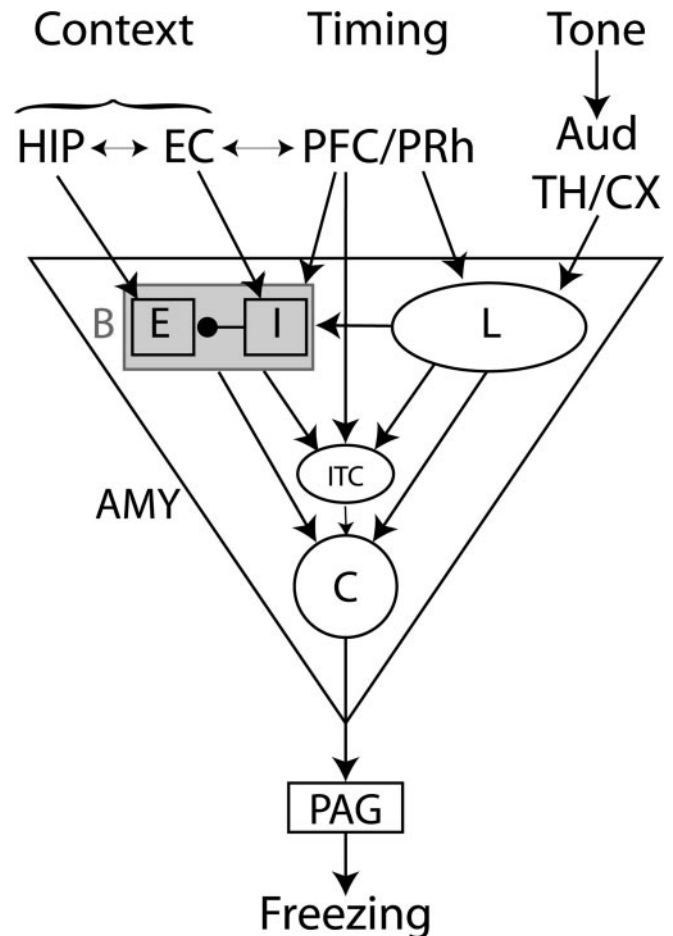


**Figure 3.** Blockade of ERK-mediated plasticity in the EC results in increased freezing and anticipatory behavior. *A*, Unique infusion sites for all behavioral experiments shown on coronal atlas plates adapted from Swanson (2004). Freezing behavior during the context-retention test after intra-EC PD098059 or vehicle infusion 40 min after training, shown binned into 30 sec time windows (PD098059,  $n = 6$ ; vehicle,  $n = 7$ ) (*B*) and after intra-EC PD098059, UO126, or vehicle infusion 40 min after training (PD098059,  $n = 15$ ; UO126,  $n = 7$ ; vehicle,  $n = 14$ ) (*C*). Control animals given six training trials demonstrate increased freezing and anticipatory freezing similar to EC-drug-infused animals ( $n = 12$ ) (*D*). Intra-EC infusion of PD098059 10 min after training does not affect freezing behavior during retention testing (PD098059,  $n = 8$ ; vehicle,  $n = 8$ ) (*E*). TR, Transition area. Data indicate mean  $\pm$  SEM; \* $p < 0.05$ .

novel context (PD098059, 0.0%; vehicle, 0.0%, NS) during the long-term retention test, and neither group demonstrated anticipatory freezing behavior (Fig. 3*E*).

## Discussion

The results presented in this report demonstrate a role for the EC in the storage of context-specific fear. Specifically, two key findings are presented. First, ERK-mediated plasticity in the EC exerts a negative effect on long-term contextual fear memory. Fear conditioning increased the number of phospho-ERK-positive cells in the EC 90 min after training. Blockade of ERK activity in the EC at this time point, but not at an earlier time point, resulted in in-



**Figure 4.** Simplified diagram of the circuitry underlying the freezing response. During fear conditioning, contextual information from the hippocampus (HIP) and EC is associated with the shock in the basal nucleus (B) of the amygdala (AMY). The perirhinal (PRh) cortex and prefrontal cortex (PFC) are thought to be involved in timing and input onto excitatory neurons (E) in the basal and lateral (L) nuclei and onto inhibitory GABAergic intercalated cells (ITC). Tone information enters L via the auditory thalamus and cortex (Aud TH/CX). The central nucleus (C) controls freezing behavior via activation of neurons in the periaqueductal gray (PAG). Blockade of ERK activity in the EC could decrease feedforward inhibition via decreased activation of inhibitory interneurons (I) in B, leading to increased freezing. Intra-EC ERK blockade could also indirectly increase the influence of PRh and PFC inputs on behavior, leading to anticipatory freezing. Arrows indicate excitatory connections, and the filled circle indicates inhibitory connections.

creased freezing to the context, which was not attributable to suppressed motor activity or generalized increases in freezing. Second, this memory enhancement contained a temporal component and mimicked the effects of additional training.

The present study does not demonstrate the mechanism by which blockade of ERK-mediated plasticity in the EC enhances contextual freezing behavior. However, EC lesions have been shown to increase fear behavior in monkeys (Meunier and Bachevalier, 2002), and EC efferents synapse onto inhibitory neurons in the basal nucleus of the amygdala (Mello et al., 1992), which is the proposed site of context-shock associations (Fanselow and LeDoux, 1999). Therefore, it is plausible that blockade of plasticity in the EC reduces feedforward inhibition onto the basal nucleus, increasing activity in the central nucleus of the amygdala, which would enhance freezing to the context (Fig. 4). Consistent with this possibility, infusions of muscimol (a

GABA<sub>A</sub> receptor agonist) into the basal and lateral nuclei abolished the memory enhancement caused by EC lesions in a conditioned odor-aversion task (Ferry et al., 1999), and inhibitory input onto the DG from the EC has also been observed (Reeves et al., 1997).

The most interesting aspect of the results presented in this report is that blockade of ERK activity in the EC resulted in anticipatory freezing; initial low levels of freezing that steadily increased and reached a maximum at the time the shock occurred during training. A well-timed response, defined as maximal responding timed to the presentation of a stimulus, usually requires extensive training (Fendt and Fanselow, 1999). Consistent with this, naive animals given six training trials demonstrated anticipatory freezing, an effect mimicked by intra-EC ERK inhibition after only two training trials. This suggests that plasticity within the EC inhibits the long-term expression of anticipatory freezing behavior after limited training. It has been proposed that a well-timed response may be mediated by perirhinal–amygdala (Mello et al., 1992) and/or prefrontal–amygdala (Brody et al., 2003) circuits (Fig. 4). The prefrontal, perirhinal, and entorhinal cortices input onto the amygdala and may compete for control of behavior (Fig. 4). Therefore, blocking ERK-mediated plasticity in the EC may unmask the influence of prefrontal–perirhinal cortices, resulting in anticipatory freezing behavior. Additional training could increase the relative contribution of prefrontal–perirhinal inputs into the amygdala until they out-compete the influence of EC inhibition.

Based on the extent of drug diffusion and the training-related increases in phospho-ERK seen throughout the EC, we cannot determine whether disruption of plasticity within the MEA or the LEA was responsible for the behavioral effects observed. However, it is thought that the MEA and LEA process different information (Witter et al., 2000), with the novelty of individual objects processed in the LEA, which has reciprocal projections preferentially with the lateral nucleus, and with spatial arrangements of objects processed in the MEA, which has reciprocal projections preferentially with the basal nucleus (Pitkanen et al., 2000). This suggests that the LEA may be more involved in cue fear memory and that the MEA may be more involved in contextual fear memory. Although additional research would be required to determine the precise roles of each EC subregion, our results provide novel insight into the role of EC plasticity in modulating contextual fear and suggest that dynamic competing processes control the expression of anticipatory freezing behavior.

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