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Bilateral phosphorylation of ERK in the lateral and centrolateral amygdala during unilateral storage of fear memories

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Abstract

We previously showed that when rats were trained to fear an auditory conditioned stimulus (CS) by pairing it with a mild unilateral shock to the eyelid (the unconditioned stimulus, or US), conditioned freezing depended upon the amygdala contralateral but not ipsilateral from the US. It was proposed that convergent activation of amygdala neurons by the CS and US occurred mainly in the amygdala contralateral from US delivery, causing memories of the CS-US association to be stored primarily by that hemisphere. In the present study, we further tested this interpretation by administering unilateral infusions of U0126 (in 50% DMSO vehicle) to block phosphorylation of extracellular signal-responsive kinase (ERK) in the amygdala prior to CS-US pairings. Conditioned freezing was impaired 24 h after training when U0126 was infused contralaterally—but not ipsilaterally—from the US, suggesting that fear memories were consolidated mainly by the contralateral amygdala. However, immunostaining experiments revealed that ERK phosphorylation was elevated in both hemispheres of the amygdala's lateral (LA) and centrolateral (CeL) nuclei after paired (but not unpaired) presentations of the CS and US. Thus, fear acquisition induced ERK phosphorylation bilaterally in the amygdala, even though the ipsilateral hemisphere did not appear to participate in conditioned freezing. These findings suggest that associative plasticity may occur in both amygdala hemispheres even when only one hemisphere is involved in freezing behavior. Conditioning-induced ERK phosphorylation was identical in both hemispheres of LA, but was slightly greater in the contralateral than ipsilateral hemisphere of CeL. Hence, asymmetric induction of plasticity in CeL might help to explain why conditioned freezing depends preferentially upon the amygdala contralateral from the US in our fear conditioning paradigm.

Keywords

mitogen-activated protein kinase; MAPK; consolidation; freezing; long-term potentiation; LTP

Introduction

Fear conditioning is an associative learning task in which subjects are trained to fear a neutral CS by pairing it with an aversive US. Evidence from rodent studies indicates that during acquisition of fear conditioning, memories of the association between the auditory CS and aversive US are stored by synaptic plasticity in the LA and central (Ce) nuclei of the amygdala (Davis, 1992; LeDoux, 2000; Schafe et al., 2005; Wilensky et al., 2006; Rabinak & Maren,

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2008; Zimmerman et al., 2007). These amygdala subnuclei are thought to receive convergent sensory information about the CS and US, so that simultaneous activation of both input pathways can trigger Hebbian strengthening of CS inputs to amygdala neurons, thereby allowing the CS to elicit fear responses after it has been paired with the US (Blair et al., 2001; Maren, 2001). It has been shown that ERK phosphorylation in the amygdala is necessary for long-term retention of behavioral fear conditioning (Schafe et al., 2000; Apergis-Schoute et al., 2005; Calandreau et al., 2006), and also for long-term maintenance of Hebbian long-term potentiation (LTP) at amygdala synapses (Huang et al., 2000; Schafe et al., 2000; Apergis-Schoute et al., 2005; Schafe et al., 2008). These findings indicate that ERK phosphorylation is likely to be a critical step in the consolidation of associative plasticity in the amygdala (Schafe et al., 2001; Sweatt, 2004).

In a previous study, we showed that when rats were trained to fear an auditory CS by pairing it with an aversive US delivered unilaterally to one eyelid, acquisition and expression of conditioned freezing was impaired by pharmacological inactivation of the amygdala contralateral but not ipsilateral from the US (Blair et al., 2005a). Based on these results, it was proposed that sensory pathways which relay the US from the eyelid to the amygdala might be lateralized, so that when the US is delivered to one eyelid, CS-US convergence (and therefore, storage of fear memories by Hebbian plasticity) occurs only in the amygdala hemisphere contralateral from the US. To further test this hypothesis, the present study examined the effects of unilaterally inhibiting ERK phosphorylation in one hemisphere of the amygdala during fear conditioning. We found that acquisition of fear conditioning was impaired by disruption of ERK phosphorylation in the amygdala contralateral but not ipsilateral from the US, supporting the idea that associative plasticity occurs mainly in the contralateral hemisphere. However, immunostaining experiments revealed that phospho-ERK expression was elevated in both hemispheres of the lateral (LA) and centrolateral (CeL) nuclei after paired (but not unpaired) presentations of the CS and US. This suggests that even though only the contralateral hemisphere is required for conditioned freezing to the CS, convergence of CS and US information may occur in both amygdala hemispheres, especially in the LA and CeL nuclei. Based on these findings, it is proposed that different forms of associative plasticity may occur in the amygdala contralateral versus ipsilateral from the site of US delivery.

Methods

All experimental procedures were approved by the UCLA Animal Research Committee and were conducted in accordance with federal guidelines for animal research.

Subjects and Surgery

Male Long-Evans rats weighing 350-400 g were reduced to 85% of their ad-lib weight through limited daily feeding. Under deep isoflurane anesthesia, intracranial infusion cannulae (22 gauge guides containing 28 gauge injectors) were implanted to position the injector tips in the lateral nucleus of the amygdala at coordinates 3.0 mm posterior, ± 5.3 mm lateral, 8.0 mm dorsal to bregma. After placement of the guide cannulae was complete, they were filled with dummy cannulae to prevent clogging between surgery and drug infusions. During the surgery, two very thin stainless steel wires (75 μ m diameter, stripped of insulation 2mm from the tip) were threaded through the skin of each eyelid for delivery of the mild periorbital shock US through a connector affixed to the skull. Periorbital stimulating wires were so thin that they could not be felt or detected by the rat except during the very brief (2 ms) current pulses. Pairs of wires were bilaterally implanted in all of the rats as a precaution against post-surgical failures of one of the electrical connections, but the US was only delivered to one eyelid in each rat during the experiment (see below). All implants were secured in place with bone cement and anchoring screws at the conclusion of the surgery.

Fear conditioning apparatus and procedure

Fear conditioning was conducted while rats foraged freely for food pellets on an elevated platform, as described in a previous study (Blair et al., 2005a). The foraging task provided a baseline of motor activity against which stimulus-evoked freezing behavior could easily be measured by an overhead video tracking system (see below). The CS was a series of 20 white noise pips (70 dB), each 250 ms in duration, presented at a rate of 1 Hz (total CS duration = 19.25 s). The US was a train of 20 very brief electrical pulses (2.5 mA), each only 2 ms in duration, delivered to the eyelid at a rate of 10 Hz (total US duration = 2 s). During paired training sessions, the US began 300 ms after the offset of the final CS pip. Thus for behavioral scoring, the CS period lasted from the onset of the first CS pip to the onset of the US (a total of 19.55 seconds). For unpaired training sessions in the immunostaining experiment, the US was delivered exactly halfway through the intertrial interval separating one trial from the next. For CS only training session in the immunostaining experiment, 16 CSs were delivered in the absence of any US. The duration of the intertrial interval for all training and testing sessions was uniformly randomized between 2-4 min on every trial. The rat's moment-to-moment position in the chamber was sampled at 30 Hz by an overhead video tracking system (Neuralynx Corporation, Bozeman, MT), which monitored the location of three light-emitting diodes (red, blue, green) attached to the animal's headstage for automated scoring of freezing and head movements using software developed in our laboratory (Moita et al., 2003, 2004; Blair et al., 2005a,b).

Experimental Design

Acquisition Experiments—After at least 5 days of recovery from surgery, rats were pre-exposed for 5 days (15 min/day) to the experimental platform, during which they learned to forage for food pellets. On day 1 of the experiment, each rat was given a test session consisting of 6 presentations of the CS alone, during which baseline freezing was measured. Some rats ($n=8$) froze for more than 7 seconds during the CS or CX periods on day 1, apparently exhibiting unconditioned fear of the novel CS before it had been paired with the US. These “pre-freezing” rats were given an additional test session prior to conditioning on day 2. Freezing scores were much lower in these rats during the day 2 test session, (indicating that their unconditioned fear of the CS had habituated), so the lowered day 2 freezing scores were used as the pre-conditioning baseline for these rats. After the baseline test sessions, rats were sorted into two balanced groups of 13 rats each, in which baseline freezing scores were similar for the two groups (the number of pre-freezing rats was also balanced between the groups). Before the training session, the CONTRA group received infusions of U0126 (1.0 μ g in 0.5 μ l of 50% DMSO/ACSF) into the amygdala hemisphere contralateral from the US, and infusions of vehicle solution (50% DMSO/ACSF) ipsilateral from the US. Conversely, the IPSI group received U0126 in the amygdala ipsilateral from the US, and vehicle in the contralateral amygdala. After a post-infusion delay of 20 minutes, all rats received a training session consisting of 16 CS-US pairings. In both the CONTRA and IPSI groups, the US was delivered to the left eyelid of 7 rats and to the right eyelid of 6 rats, so the side of US delivery was counterbalanced within each group. Twenty-four hours (24 h) after training (on day 3), each rat was given a test session (6 presentations of the CS alone) to measure conditioned freezing responses.

Expression Experiments—Some of the rats (16 out of 23, with 8 trained on the left eyelid and 8 trained on the right eyelid) received additional overtraining, beginning with 16 CS-US pairings immediately after the test session on day 3. These rats received 6 CS alone trials followed by 16 CS-US pairings on five subsequent days (days 4-8). On day 9, all of the rats received infusions of U0126 into the right hemisphere and DMSO vehicle into the left hemisphere, so the drug was delivered contralaterally for rats trained on the left eyelid and ipsilaterally for rats trained on the right eyelid. After a post-infusion delay of 20 minutes, the

rats were given a test session of 6 CS alone trials. On the two following days (days 10 and 11), the rats received a standard regimen of 6 CS alone trials followed by 16 CS-US pairings. On day 12, all of the rats received infusions of U0126 into the left hemisphere and DMSO vehicle into the right hemisphere, so the drug was delivered contralaterally for rats trained on the right eyelid and ipsilaterally for rats trained on the left eyelid. After a post-infusion delay of 20 minutes, the rats were given a test session of 6 CS alone trials. Using this repeated measures design, each rat received pre-test infusions of U0126 into both the ipsilateral and contralateral amygdala hemisphere on different days (day 9 or day 12) in counterbalanced order.

U0126 / Vehicle Infusions

U0126 (Sigma, St. Louis, MO) was dissolved in 100% DMSO to a stock concentration of 4 ug/ul. For the pre-conditioning and pre-expression infusions, the stock was then diluted 1:1 in ACSF. Vehicle was prepared by diluting 100% DMSO 1:1 in ACSF. For all experiments, drug or vehicle were infused through a 28-gauge (amygdala) injector cannulae attached to a 1.0 µl Hamilton syringe via polyurethane tubing as described in a previous study (Blair et al., 2005a). Into each hemisphere, 0.5 µl of fluid (drug or vehicle) was infused at a rate of 0.25 µl /min. After each infusion, the cannulae were left in place for an additional 1-2 min to allow diffusion of the drug away from the cannulae tip, after which the injectors were removed and replaced with dummy cannulae.

Immunohistochemistry

For immunohistochemistry, rats were handled and pre-exposed to the experimental platform and pellet chasing task for 5 days, exactly as in the U0126 experiments. Following pre-exposure they received either paired, unpaired, or CS alone sessions (see above under Experimental Design). Each of these sessions lasted 50-55 minutes because of random variability in the intertrial intervals. At a time point 65 minutes after the first US presentation (10-15 minutes after the end of the training session) each rat was rapidly anaesthetized with isoflurane (to prevent injection-induced stress) and then intraperitoneally injected with a 25 mg lethal dose of sodium pentobarbital. The 65-minute time point was chosen based upon prior studies showing that auditory fear conditioning significantly elevated phospho-ERK in the amygdala at 60 minutes (but not 30 or 180 minutes) following a single CS-US pairing (Schafe et al., 2000). Under deep anesthesia, rats were intra-cardially perfused with ice-cold PBS, followed by ice-cold 4% paraformaldehyde in PBS. Brains were removed and post-fixed overnight and then cryoprotected in 30% sucrose in PBS. When the brains sunk in the cryoprotectant, 40 µm slices of the amygdala were taken on a cryostat at coordinates ranging from -1.8 to -3.6 mm posterior to bregma. Every other section was preserved, to prevent double-counting of phospho-ERK stained cells in adjacent sections. Immunohistochemistry was performed for control and experimental brain sections using the free floating method as described previously by (Eitan et al., 2003). Briefly, sections were incubated overnight with antibodies against phospho-p42/44 ERK (Cell Signalling Technology, Beverly, MA) diluted 1:300 at 4°C. Sections were then rinsed and incubated with biotin-conjugated anti-rabbit antibodies followed by Avidin-Biotin Complex enhancement (Vector Laboratories, Burlingame, CA). Staining was revealed using 3,3'-diaminobenzidine (DAB) tablets (Fluka, Steinheim, Switzerland) and sections were mounted on subbed slides and coverslipped. Matched sections from comparable rostro-caudal levels of the amygdala in each hemisphere were used for quantifying phospho-ERK labeling. Sections were digitally photographed at 4× magnification under brightfield illumination. Boundaries of specific amygdala subnuclei were determined by overlaying the photographs with Adobe Illustrator templates from the atlas of Paxinos and Watson (1998) at the appropriate rostrocaudal coordinate level, and editing the template boundaries of each subnucleus to match the underlying photograph. Phospho-ERK positive cells were separated from the background using a uniform standard for digital thresholding of grayscale images in

Adobe photoshop, and counting of the resulting puncta was performed using ImageJ (National Institutes of Health, USA).

Results

Effects of unilateral phospho-ERK inhibition on fear conditioning

A total of 26 rats were surgically implanted with bilateral amygdala infusion cannulae (Figure 1a shows histological reconstructions of cannula tip placements). The rats were subdivided into two groups, CONTRA (n=13) and IPSI (n=13), which received pre-training infusions of U0126 into the amygdala contralateral versus ipsilateral from the US, respectively (see Methods). All rats received simultaneous vehicle infusions into the hemisphere opposite from the drug hemisphere. Although injector tips were targeted at the lateral nucleus of the amygdala, the infusion volume (0.5 μ l per side) was probably large enough for the drug to diffuse into neighboring regions, including the basal and central amygdala nuclei.

Fear acquisition is impaired by contralateral but not ipsilateral U0126—Figure 2 shows averaged freezing scores from the IPSI (Figure 2a) and CONTRA (Figure 2b) groups during pre- versus post-conditioning test sessions. A $2 \times 2 \times 2$ ANOVA was performed on these freezing scores with session (Pre- vs. Post-Training, repeated), stimulus (CX vs. CS, repeated), and group (IPSI vs. CONTRA, independent) as factors. There were significant main effects of stimulus ($F_{1,24}=16.06$, $p=.0005$) and session ($F_{1,24}=5.04$, $p=.008$), but there was no main effect of group ($F_{1,24}=0.03$, $p=.856$). However, a significant interaction between group and stimulus ($F_{1,24}=7.25$, $p=.013$) and a significant three-way interaction effect ($F_{1,24}=5.47$, $p=.028$) indicated that conditioned freezing responses were sensitive to all three factors, including group. Newman-Keuls posthoc tests revealed that in the IPSI group, freezing to the post-conditioning CS was significantly elevated relative to the post-conditioning CX ($p=.0008$), pre-conditioning CX ($p=.0003$), and pre-conditioning CS ($p=.0015$). By contrast, in the CONTRA group, freezing during the post-conditioning CS was not elevated with respect to the post-conditioning CX ($p=.99$), pre-conditioning CX ($p=.23$), or pre-conditioning CS ($p=.42$). Since rats in the IPSI group acquired freezing responses to the CS after training, but rats in the CONTRA group did not, it appears that pre-training infusions of U0126 into the contralateral but not ipsilateral amygdala impaired acquisition of conditioned freezing.

Unconditioned responses to the US—Evidence suggests that disruptions of the amygdala can attenuate the aversiveness of noxious stimuli (Borcz & Leaton, 2003; Blair et al., 2005b), so we examined whether unconditioned responses to the US were differentially affected by infusions of U0126 into the contralateral versus ipsilateral amygdala. Figure 2d shows unconditioned motor responses (head-jerking movements) elicited by the eyelid US during training for each group, measured by comparing head speed during the CX versus US (see Blair et al., 2005b). A 2×2 ANOVA revealed a main effect of stimulus (CX vs. US: $F_{1,24}=22.5$, $p=.00008$) but not of group (IPSI vs. CONTRA: $F_{1,24}=0.36$, $p=.55$) and no stimulus-by-group interaction ($F_{1,24}=1.73$, $p=.2$). A Newman-Keuls posthoc test revealed that unconditioned responses to the US were not significantly different in the IPSI versus CONTRA groups ($p=.16$). Thus, impaired fear conditioning in the CONTRA group was not attributable to impaired US sensitivity in this group.

Effects of Infusions on Fear Expression—To assess how U0126 affected expression of conditioned freezing, 16 of the 26 rats (8 from the IPSI group and 8 from the CONTRA group) were overtrained after the initial training and test sessions. Overtrained rats then received pre-test infusions of U0126 into the amygdala ipsilateral from the US on one test day, and contralateral from the US on another test day (in counterbalanced order), with DMSO vehicle always infused into the non-drug hemisphere (see Methods). Using this repeated measures

approach, we examined how freezing to the CX and CS were affected by pre-test U0126 infusions into the ipsilateral versus contralateral hemisphere of the 16 overtrained rats (Figure 2c). A 2×2 ANOVA revealed a main effect of stimulus (CX vs. CS: $F_{1,15}=11.9$, $p=.0035$) but not of hemisphere (ipsi vs. contra: $F_{1,15}=0.16$, $p=.69$) and no stimulus-by-hemisphere interaction ($F_{1,24}=0.87$, $p=.36$). These results suggest that in well-trained rats, CS-evoked freezing remained intact after pre-test infusions of U0126 into either the ipsilateral or contralateral amygdala hemisphere. However, Newman-Keuls posthoc tests failed to detect that freezing to the CS was significantly elevated above the CX baseline after pre-test infusions into only the ipsilateral ($p=.11$) or contralateral ($p=.28$) hemispheres. The lack of significance for within-hemisphere freezing to the CS appeared to be caused by elevated baseline freezing to the CX after the infusions, rather than by diminished freezing to the CS. As explained in the Methods section, pellet-chasing behavior provided a constant background of motor activity against which freezing responses could be detected by our video tracking system. Hence, the increased CX freezing that was observed after DMSO infusions may have been related to decreased motivation to perform the pellet-chasing task after DMSO infusions.

To further test this, a group of rats ($n=6$) was bilaterally implanted with infusion cannula and trained to chase pellets over 5 days of platform pre-exposure (exactly as described in Methods for rats in the fear conditioning groups). On day 6, these rats received bilateral infusions of DMSO vehicle prior to pellet-chasing (they were never fear conditioned). After a 1 h post-infusion delay, rats were given a 15-minute session of pellet chasing on the platform. The rats then rested in their home cages until a 3 h after the infusion had been given, at which time they were given another 15-minute pellet chasing session. The rats then returned to the vivarium until the following day, when they were given a final pellet-chasing session 24 h after the infusion. To quantify behavioral effects of the infusions, we calculated the percentage of time throughout the pellet-chasing sessions during which the rats met the immobility criterion (see Methods) that was used to score freezing in fear-conditioning sessions (Figure 2e). A one-way ANOVA of freezing percentages with session as the repeated factor revealed a significant main effect of DMSO infusion ($F_{3,15}=4.89$, $p=.014$). Post-hoc comparisons using the Newman-Keuls test revealed that compared to the drug-free session on day 5 (the last day of pre-exposure), freezing on day 6 was elevated 1 h ($p=.04$) and 3 h ($p=.02$) after the infusion. On day 7 (24 h after the infusion), the freezing percentage was no longer different from the day 5 baseline ($p=.94$). These results indicate that DMSO alone had an effect on the rats' movement behavior, but do not explain why. One possibility is that DMSO infusions into the amygdala induced fear and anxiety in the rats, leading to elevated baseline freezing expression. Another possibility is that DMSO altered the rats' motivation to perform the pellet-chasing task. DMSO-infused rats continued to perform exploratory movements on the platform, but they appeared to consume fewer food pellets than non-infused rats, so it is possible that temporary appetite suppression may have been a side effect of infusing the DMSO vehicle into the amygdala.

Since baseline motor activity was reduced (and thus, baseline freezing was increased) in rats that had recently been infused with the DMSO vehicle, it was difficult to assess whether the effects of pre-test infusions were specific to the expression of CS-evoked freezing, or were instead attributable to a more general influence on motor behavior. Because of this confound, it was not fully clear how U0126 affected expression of conditioned freezing. In addition, it was not possible to conduct short-term expression tests in rats that had been given pre-training infusions of U0126, because baseline motor activity was shifted during the time window for testing short-term retention. Despite these difficulties with controlling for expression effects, our behavioral findings clearly showed that when rats were trained to fear a CS that predicted a unilateral US, the contribution of ERK phosphorylation to acquisition (and possibly expression) of fear conditioning was lateralized to the amygdala contralateral from the US (Figures 2a and 2b).

Induction of ERK phosphorylation during fear conditioning

To further investigate lateralization of ERK signaling in the amygdala, we performed immunostaining experiments to examine whether phosphorylation of ERK occurred preferentially in the amygdala contralateral from the US. A total of 27 rats were bilaterally implanted with periorbital wires for US delivery. Each rat received 5 days of pre-exposure to the behavior chamber (and training to perform the pellet chasing task), exactly like the rats in the behavior studies presented above. Following pre-exposure, the rats were divided into three groups. The PAIR group (n=11) received a training session consisting of 16 CS-US pairings, identical to the training sessions described above for behavioral experiments. The UNPAIR group (n=8) received 16 CS presentations that were explicitly unpaired with 16 US presentations (see Methods); Blair et al. (2005a) have shown that such unpaired training produces no conditioned freezing to the CS. The CS ONLY group (n=8) received 16 identical CS presentations, but the US was never delivered to this group. In all groups, the US was delivered on the left side for half of the rats and the right side for the other half, except for the PAIR group in which had an odd number of rats, so 5 rats received the US on the left and 6 on the right. The training sessions lasted 50-55 minutes for each rat (because of the random intertrial interval). At a time point 65 minutes after the start of the training session (10-15 minutes after the end of the session), rats were deeply anesthetized with sodium pentobarbital and perfused intracardially with 4% paraformaldehyde in PBS (see Methods). Following perfusions, brains were fixed and sectioned throughout the rostrocaudal extent of the amygdala (from 1.8 to 3.8 mm posterior to bregma), and sections were immunolabeled for phospho-ERK using DAB (Eitan et al., 2003). Using boundaries from the atlas of Paxinos & Watson (1998), immunolabeled cells were quantified separately within four distinct subnuclei of the amygdala: the lateral nucleus (LA), basal nucleus (B), centromedial nucleus (CeM), and centrolateral nucleus (CeL). Average cell counts (per mm²) in each of these four areas (separated by hemisphere) are shown in Figure 3.

LA nucleus—A one-way ANOVA of labeled cells in the LA nucleus (from both hemispheres combined) revealed a significant main effect of group ($F_{2,51}=4.78$, $p=.012$), and a Newman-Keuls posthoc test revealed that the number of labeled cells was significantly higher in the PAIR group than in the CS ONLY ($p=.016$) or the UNPAIR ($p=.045$) groups, but not higher in the UNPAIR than the CS ONLY group ($p=.43$). The number of labeled cells in LA did not differ ipsilaterally versus contralaterally from the US in the PAIR ($t_{10}=0.06$, $p=.95$) or UNPAIR ($t_7=0.79$, $p=.46$) group, indicating that ERK phosphorylation was not hemispherically lateralized with respect to the US. In the PAIR group, cell counts in LA were significantly elevated in both hemispheres (ipsi: $t_{25}=2.46$, $p=.02$; contra: $t_{25}=2.53$, $p=.018$) when compared against cell counts (n=16) from both hemispheres combined in the CS ONLY group. By contrast, cell counts were not elevated in either hemisphere for the UNPAIR group (ipsi: $t_{22}=55$, $p=.59$; contra: $t_{22}=1.45$, $p=.16$). Hence, ERK phosphorylation was induced in both hemispheres of LA after paired but not unpaired presentations of the CS and US.

B nucleus—A one-way ANOVA of labeled cells in the B nucleus (from both hemispheres combined) revealed no effect of group ($F_{2,51}=0.29$, $p=.75$). In addition, the number of labeled cells did not differ between the ipsilateral and contralateral hemispheres in the PAIR ($t_{10}=.41$, $p=.69$) or UNPAIR ($t_7=.45$, $p=.66$) groups. In the PAIR group, cell counts in LA were not elevated in either hemisphere (ipsi: $t_{25}=0.93$, $p=.36$; contra: $t_{25}=0.59$, $p=.56$) when compared against both combined hemispheres combined in the CS ONLY group. Cell counts also were not elevated in either hemisphere for the UNPAIR group (ipsi: $t_{22}=0.41$, $p=.69$; contra: $t_{22}=0.58$, $p=.57$). These results indicate that phospho-ERK labeling in the B nucleus was not detectably affected by fear conditioning.

CeL nucleus—A one-way ANOVA of labeled cells in CeL (from both hemispheres combined) revealed a significant effect of group ($F_{2,51}=6.27$, $p=.004$), and a Newman-Keuls posthoc test revealed that the number of labeled cells was significantly higher in the PAIR group than in the CS ONLY ($p=.006$) or UNPAIR ($p=.016$) groups, but not higher in the UNPAIR than the CS ONLY group ($p=.47$). Hence, it appears that ERK phosphorylation in CeL was induced by paired but not unpaired presentations of the CS and US. The number of labeled cells in CeL was larger in the contralateral than in the ipsilateral hemisphere for both the PAIR and UNPAIR groups, but this difference did not reach significance in either group (PAIR: $t_{10}=1.56$, $p=.15$; UNPAIR $t_7=1.2$, $p=.27$). When compared against both combined hemispheres from the CS ONLY group, cell counts were significantly elevated in both hemispheres of the PAIR group (ipsi: $t_{25}=2.52$, $p=.018$; contra: $t_{25}=3.21$, $p=.004$), but were not significantly elevated in either hemisphere of the UNPAIR group (ipsi: $t_{22}=0.42$, $p=.68$; contra: $t_{22}=0.83$, $p=.42$).

Since the PAIR group showed a possible trend for enhanced immunostaining in the contralateral hemisphere of CeL, cell counts in the PAIR group were compared against both hemispheres combined from the UNPAIR group. This analysis indicated that immunostaining in the PAIR group was significantly elevated in the contralateral ($t_{25}=2.43$, $p=.02$) but not the ipsilateral ($t_{25}=1.65$, $p=.11$) hemisphere. This was the only evidence obtained from any of our analyses which suggested the possibility of enhanced ERK phosphorylation in the hemisphere contralateral from the US.

CeM nucleus—A one-way ANOVA of labeled cells in the CeM nucleus (from both hemispheres combined) revealed no effect of group ($F_{2,51}=0.56$, $p=.57$). In addition, the number of labeled cells did not differ between the ipsilateral and contralateral hemispheres in the PAIR ($t_{10}=0.02$, $p=.99$) or UNPAIR ($t_7=.87$, $p=.41$) groups. In the PAIR group, cell counts in LA were not elevated in either hemisphere (ipsi: $t_{25}=0.82$, $p=.42$; contra: $t_{25}=0.9$, $p=.38$) when compared against both combined hemispheres combined in the CS ONLY group. Cell counts also were not elevated in either hemisphere for the UNPAIR group (ipsi: $t_{22}=0.56$, $p=.58$; contra: $t_{22}=0.23$, $p=.82$). These results indicate that phospho-ERK labeling in the CeM nucleus was not detectably affected by fear conditioning.

Discussion

Aversive stimuli are thought to instruct fear conditioning by inducing associative LTP at glutamatergic synapses in the amygdala and elsewhere (LeDoux, 2000; Maren, 2001; Seymour and Dolan, 2008). ERK phosphorylation is a key step in the long-term consolidation of associative LTP (Bading and Greenberg, 1991; Martin et al., 1997; Sweatt, 2004; Thomas and Huganir, 2004), and prior studies of auditory fear conditioning (using a footshock US) have shown that pre-training infusions of U0126 into the amygdala impaired conditioned freezing when rats were tested 24 h after training, but not at shorter retention intervals (Schafe et al., 2000; Apergis-Schoute et al., 2005; Calendreau et al., 2006). In addition, neurophysiological studies have shown that ERK signaling is needed for maintaining late-phase but not early-phase LTP at amygdala synapses (Huang et al., 2000; Schafe et al., 2000; Apergis-Schoute et al., 2005; Schafe et al., 2008). Taken together, these prior findings suggest that ERK phosphorylation is likely to be required for long-term maintenance but not early induction of associative plasticity in the amygdala during fear conditioning. The results of our present experiments add to this evidence by providing new clues about the causal relationship between ERK signaling and behavioral learning, and also about which subnuclei of the amygdala might support ERK-dependent plasticity during fear conditioning.

ERK-dependent memory consolidation during fear conditioning

Here we found that when an auditory CS was paired with an aversive US delivered unilaterally to one eyelid, CS-evoked freezing depended upon ERK signaling in the amygdala contralateral but not ipsilateral from the US. These results are consistent with our previous data showing that conditioned freezing depended upon neural activity in the amygdala contralateral but not ipsilateral from the US during the same fear conditioning paradigm (Blair et al., 2005a). One possible explanation for this pattern of findings is that when fear conditioning is instructed by a unilateral eyelid shock US (as in our present and prior studies), freezing behavior depends primarily upon synaptic plasticity (and subsequent ERK-mediated memory consolidation) that occurs in the amygdala contralateral—but not ipsilateral—from the US.

Comparison with other fear conditioning paradigms—The average levels of conditioned freezing in our experiments are lower than in prior studies that have investigated the role of ERK signaling in fear conditioning. Several factors are likely to contribute to the lower freezing levels observed in our fear conditioning paradigm. First, in our studies the US is delivered unilaterally to a very small and highly localized patch of skin on the eyelid, rather than bilaterally to the pads of all four feet. Second, in our studies freezing is scored by a video tracking system which measures suppression of movement during a pellet-chasing task on an open platform, rather than by human observers or infrared detectors that score movement during free exploration in an enclosed chamber. Third, in our studies the CS is a series of white noise pips rather than a continuous tone, and the onset of the pips can sometimes elicit startle-like reflex responses that momentarily interrupt freezing behavior (see Moita et al., 2003). In prior studies using a bilateral footshock as the US, it has been reported that unilateral amygdala lesions cause only mild impairment of conditioned freezing to an auditory CS in rats, with no significant difference between the left and right hemispheres (LaBar and LeDoux, 1996; Goossens and Maren, 2001; Baker and Kim, 2004). If our task yielded higher freezing levels, we might observe some spared freezing after disruptions of neural activity or ERK signaling in the amygdala contralateral from this shock, rather than abolition of freezing. Based on the possibility of such a “floor effect,” it may be debated whether the freezing responses acquired in our fear conditioning paradigm are exclusively dependent upon the amygdala contralateral from the US, or merely biased to depend more upon the contralateral than the ipsilateral hemisphere. Nonetheless, our present and prior findings provide clear evidence that when an aversive US is delivered unilaterally to one eyelid, conditioned freezing depends preferentially upon the amygdala contralateral from the US. The degree of this hemispheric bias has not been fully characterized, and should be clarified by future studies.

Role of ERK signaling in acquisition versus expression of freezing—ERK signaling can regulate neural excitability as well as plasticity (Yuan et al., 2006), so it cannot be ruled out that U0126 may have affected expression as well as acquisition of conditioned fear. Unfortunately, baseline motor activity (food foraging) was shifted for several hours after bilateral infusions of the DMSO vehicle into the amygdala. This made it difficult to determine whether pre-test infusions of U0126 affected expression of conditioned freezing responses, and also prevented us from testing whether U0126 spared freezing at short retention intervals after fear conditioning. Despite these limitations, our results provide clear evidence that freezing responses were selectively impaired by pre-training infusions of U0126 into the amygdala contralateral but not ipsilateral from the US. Hence, any contributions of ERK signaling to synaptic plasticity or other cellular processes underlying freezing behavior were localized to the contralateral amygdala hemisphere.

Lateralization of US pathways to the amygdala—It has been hypothesized that convergence of sensory information about the CS and US onto single amygdala neurons can trigger Hebbian plasticity at the synapses which relay the CS to those neurons, thereby storing

a memory of the CS-US association (LeDoux et al., 2000; Blair et al., 2001; Maren, 2001). If this hypothesis is correct, then when a mild unilateral eyelid shock is used as the US for fear conditioning, nociceptive US signals might be relayed to the amygdala via lateralized sensory pathways, so that CS-US convergence (and thus, associative plasticity) occurs mainly in the amygdala contralateral from the US. Nociceptive signals from the eyelid enter the brain through the trigeminal nucleus of the medullary dorsal horn on the side ipsilateral to the eyelid, and then cross to the contralateral side via projections from the trigeminal nucleus to areas including the intralaminar thalamus and parabrachial nuclear complex, which in turn send uncrossed projections to the amygdala (LeDoux et al., 1987; Bernard et al., 1993, 1995; Feil and Herbert, 1995; Jasmin et al., 1997). The contributions of these different nociceptive pathways to fear conditioning are not fully understood (Shi and Davis, 1999; Brunzell and Kim, 2001; Lanuza et al., 2004), but their hemispheric organization suggests that they may be well suited convey information about an aversive US from the eyelid to the contralateral amygdala.

Induction of phospho-ERK immunostaining during fear conditioning

ERK phosphorylation is triggered by calcium entry through NMDA receptors, which is also the primary event that triggers many forms of associative LTP (Bading and Greenberg, 1991; Malenka and Nicoll, 1993). Hence, phospho-ERK immunostaining in post-mortem neurons may indicate that the neurons experienced an influx of NMDA calcium prior to fixation, which in turn suggests that the neurons might also have undergone NMDA-dependent synaptic plasticity. Supporting this idea, prior studies have shown that behavioral learning can induce phospho-ERK immunostaining in brain regions where NMDA-dependent plasticity is thought to occur: fear conditioning activates ERK in amygdala (Schafe et al., 2000; Gresack et al., 2009), water-maze learning activates ERK in hippocampus (Blum et al., 1999; Satoh et al., 2007), drugs of abuse activate ERK in striatum (Valjent et al., 2004; Zhang et al., 2004), and patterned visual stimulation activates ERK in the developing visual cortex (Cancedda et al., 2003).

Localization of ERK phosphorylation to LA and CeL—Here we observed that after paired (but not unpaired) CS-US presentations, phospho-ERK immunostaining was elevated specifically in the LA and CeL (but not in B or CeM), suggesting that NMDA-dependent plasticity may have occurred primarily in these two subnuclei during our fear conditioning task. This interpretation is consistent with prior evidence implicating both LA and CeL as possible sites of learning-related plasticity during fear conditioning. For example, LTP experiments have shown that NMDA-dependent plasticity occurs at synaptic inputs to both LA and CeL (Chapman et al., 1990; Clugnet & LeDoux, 1990; Huang & Kandel, 1998; Fu & Shinnick-Gallagher, 2005; Lopez de Armentia M. and Sah P., 2007), and behavioral fear conditioning depends upon NMDA receptors in the both the basolateral and central nuclear complexes of the amygdala (Miserendino et al., 1990; Kim et al., 1991; Schafe et al., 2005; Wilensky et al., 2006). It has been proposed that LA may be the primary site of learning related plasticity in the basolateral complex (LeDoux, 2000), whereas CeL could be the primary locus of plasticity in the central complex (Ehrlich et al., 2009). If so, then this would be consistent with our present observations that CS-US pairings elevated ERK phosphorylation selectively in LA and CeL.

Bilateral induction of ERK phosphorylation in the amygdala—We observed that phospho-ERK immunostaining was elevated in the amygdala ipsilateral as well as contralateral from the US, even though conditioned freezing appeared to depend only upon ERK signaling in the contralateral hemisphere. This result raises questions about the idea (discussed above) that CS-US convergence occurs preferentially in the contralateral amygdala, and also about the nature of the causal relationship between associative plasticity and ERK immunostaining. Why was ERK phosphorylation in the ipsilateral amygdala selectively activated by CS-US

pairings, if this hemisphere was not required for conditioned freezing? One possibility is that CS-US pairings induced associative plasticity only in the amygdala contralateral (and not ipsilateral) from the US, but phospho-ERK immunostaining was induced in both amygdala hemispheres because pairing-induced ERK phosphorylation was not exclusively localized to neurons that underwent associative plasticity during conditioning. Another possibility is that immunoreactive neurons in both amygdala hemispheres underwent associative plasticity (accompanied by ERK phosphorylation) during fear conditioning, but plasticity in the ipsilateral amygdala was not essential for conditioned freezing behavior. Evidence suggests that multiple forms of plasticity can occur at amygdala synapses (see Sah et al., 2008 for review). Hence, fear conditioning might induce plasticity at different groups of synapses in each hemisphere, or might induce different forms of plasticity (for example, potentiation versus depression) at the same synapses in opposite hemispheres. If so, then the synaptic changes in both hemispheres may involve ERK signaling, even though only those in the contralateral hemisphere are required for conditioned freezing behavior. Perhaps plasticity in the ipsilateral amygdala participates regulating fear-conditioned behaviors other than freezing, which were not measured here.

Lateralization of ERK signaling in LA versus CeL—Although our experiments did not yield strong evidence for lateralization of associative plasticity in the amygdala, we did see suggestive indications that phospho-ERK immunostaining might be more contralaterally biased in CeL than in LA. Rats given CS-US pairings showed significantly higher levels of phospho-ERK immunostaining in the CeL contralateral but not ipsilateral from the US, when compared against the combined hemispheres from rats that were given unpaired training. However, contralaterally biased immunostaining was not present at all in LA. This suggests the possibility that impaired fear acquisition after contralateral infusions of U0126 may have mainly resulted from blockade of ERK phosphorylation in the contralateral CeL, where the highest levels of ERK phosphorylation were seen in our experiments. It follows that if lateralized US pathways are responsible for the hemispheric bias observed in our fear conditioning paradigm, then US pathways targeting CeL might be more contralaterally biased than those targeting LA. Supporting this possibility, it has been shown that the parabrachial complex (a prominent center for nociceptive processing) sends uncrossed projections to the amygdala that specifically target CeL (Bernard et al., 1993).

Summary and conclusions

In summary, our present findings support prior evidence that when rats are trained to fear an auditory CS paired with a unilateral US, conditioned freezing depends mainly upon associative plasticity in the amygdala contralateral from the US. However, our results also suggest that this lateralization of the amygdala's role in fear conditioning might not be fully explained by unilateral convergence of the CS and US in the contralateral hemisphere, as previously proposed (Blair et al., 2005a). Instead, it appears that neurons in both amygdala hemispheres are sensitive to the CS-US contingency, and thus, the two hemispheres may make differing contributions to learned fear behaviors. To the extent that lateralized US pathways may be responsible for the reliable hemispheric bias observed in our fear conditioning paradigm, evidence from phospho-ERK immunostaining experiments suggests that US pathways targeting CeL may be more contralaterally biased than those targeting LA. Further investigation of these hemispheric differences may help to clarify how different neural subpopulations (or different forms of synaptic plasticity) in the amygdala contribute to specific types of defensive behaviors during fear conditioning.

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Figure 1.
Injection sites for infusions of U0126 and vehicle into the amygdala.

Figure 2.

Effects of U0126 infusions on acquisition and expression of conditioned freezing. **a)** Rats acquired conditioned freezing to the CS after pre-training infusions of U0126 into the amygdala ipsilateral from the US. **b)** Rats failed to acquire conditioned freezing after pre-training infusions of U0126 into the amygdala contralateral from the US. **c)** In overtrained rats, pre-test infusions of U0126 caused elevated baseline freezing to the CX, but a small amount of CS-evoked freezing was still detectable over this baseline (see text). **d)** Unconditioned responses to the US did not differ after pre-training infusions of U0126 into the amygdala ipsilateral versus contralateral from the US. **e)** Immobility scores were increased during pellet chasing for several hours after bilateral infusions of DMSO vehicle in rats that had not been fear conditioned. Symbols denote the significance of the indicated comparisons in all graphs (* $p < .05$, ** $p < .01$, *** $p < .001$, 'ns' = not significant).

Figure 3. Phospho-ERK immunostaining induced in the amygdala after paired (PAIR), unpaired (UNP), or CS only (CS) sessions. Photomicrographs at right show examples of immunostained sections from one of the rats in each group, with outlines indicating the boundaries of subnuclei. Cell counts were quantified separately in LA, B, CeL, and CeM. Bar graphs at left show mean cell counts (cells/mm²) for each subnucleus in each group, with symbols denoting results of a t-test comparing the labeled bar against the combined counts in both hemispheres of the CS ONLY group (* $p < .05$, ** $p < .01$, 'ns' = not significant).