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Conditioned turning behavior: A Pavlovian fear response expressed during the post-encounter period following aversive stimulation

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Abstract

Rats were trained to fear an auditory conditioned stimulus (CS) by pairing it with a mild electric shock (the unconditioned stimulus, or US) delivered to one eyelid. After training, the CS elicited two different conditioned fear responses from rats: a passive freezing response, and an active turning response. The balance between these two modes of conditioned responding depended upon the rat's recent history of encounters with the US. If rats had not recently encountered the US, then they responded to the CS by freezing. But after recently encountering the US, rats exhibited CS-evoked turning responses that were always directed away from the trained eyelid, even if the US had recently been delivered to the opposite (untrained) eyelid. This post-encounter turning behavior was not observed in rats that had been trained with unpaired presentations of the CS and US, indicating that even though CS-evoked turning was selectively expressed after recent encounters with the US, it was nonetheless a conditioned Pavlovian fear response that depended upon a learned association between the CS and US. Further supporting this conclusion, pharmacological inactivation experiments showed that expression of both freezing and turning behaviors depended upon lateralized circuits in the amygdala and periaqueductal gray (PAG) that are known to support expression of Pavlovian fear responses. These findings indicate that even though the ability of a CS to elicit Pavlovian fear responses depend upon the long-term history of CS-US pairings, the mode of conditioned responding (freezing versus turning in the present experiments) can be modulated by short-term factors, such as the recent history of US encounters. We discuss neural mechanisms that might mediate such short-term transitions between different modes of defensive responding, and consider how dysregulation of such mechanisms might contribute to clinical anxiety disorders.

Keywords

amygdala; midbrain; periaqueductal gray; lateralization; predatory imminence

INTRODUCTION

Most animals (including humans) are endowed with an innate repertoire of defensive responses for coping with threats to their survival. Defensive responses change as threat

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levels increase, and can thus be organized along a spectrum referred to as the “predatory imminence continuum” (Blanchard & Blanchard, 1969a,b; Bolles, 1970; Fanselow & Lester, 1988; Mobbs et al., 2007, 2009). In rats, low levels of threat are characterized by engagement in non-defensive behaviors, such as exploration or goal-seeking. At intermediate threat levels (referred to as “circa-strike”), the rat begins to perceive danger and engage in behaviors such as freezing to avoid detection by potential predators, or emitting warning calls to notify conspecifics of a possible threat. The highest threat levels (referred to as “post-encounter”) occur after the rat has suffered injury or come under attack by a predator, triggering responses such as distress calls, fleeing from danger, or fighting back against the predator if no escape is possible.

Much of what is currently known about neural circuits mediating defensive responses has been learned from rodent studies of Pavlovian fear conditioning, in which rats (or mice) are trained to fear a neutral CS by pairing it with an aversive US (for review see Davis, 1992; LeDoux, 2000). In such studies, conditioned fear is typically assessed by presenting a CS to previously trained subjects that have not recently encountered the US, while they are in a baseline state of low predatory imminence (for example, freely exploring their environment, or engaged in a task such as licking or bar-pressing). Under these testing conditions, the CS can elicit circa-strike defenses—such as freezing or startle potentiation—which are measured to index the level of conditioned fear. An underlying assumption of such experiments is that expression of the measured responses is monotonically related to the intensity of conditioned fear (that is, more responding indicates more fear). However, this monotonicity assumption may not always be valid, because if fear intensity exceeds the threshold for triggering post-encounter defensive strategies, then decreases in the expression of circa-strike behaviors (like freezing or startle) may reflect greater—not lesser—fear of the CS (see Blanchard & Blanchard, 1969a,b; Bolles, 1970; Davis & Astrachan, 1979; Fanselow & Lester, 1988). Consequently, the range of conditioned fear intensities that can be accurately indexed by Pavlovian circa-strike behaviors is constrained to remain below the threshold for expression of post-encounter defenses. This is an unfortunate limitation, because rodent fear conditioning has been widely adopted as an animal model for investigating the neurobiological basis of clinical anxiety disorders (Davis and Whalen, 2001; Rau et al., 2005; Davis et al., 2006; Milad et al. 2006; Miller & McEwen, 2006; Rauch et al., 2006). But some anxiety symptoms in human patients—such as panic attacks—may involve activation of post-encounter response systems (see Craske, 1999). Standard rodent models of fear conditioning may not recruit these post-encounter response systems, since they are based upon methods that favor the expression of circa-strike behaviors.

We have previously conducted fear conditioning experiments using a paradigm in which rats are given an auditory CS paired with a unilateral shock US delivered to one eyelid (Moita et al., 2003, 2004; Blair et al., 2005a,b; Tarpley et al. 2009; Johansen et al. 2010). During these experiments, we have observed that in addition to CS-evoked freezing behavior, well-trained rats also tend to exhibit another distinctive response to the CS: turning in circles away from the eyelid where shock is anticipated. Here, we conducted a formal investigation of this novel turning response. We report that, like freezing, CS-evoked turning behavior is a Pavlovian response which depends upon lateralized circuits in the amygdala and PAG that mediate acquisition and expression of conditioned fear (Fanselow, 1991; Bandler & DePaulis, 1991; Davis, 1992; Maren, 1998; LeDoux, 2000). But unlike freezing, the turning response is expressed selectively after recent encounters with the US, and not at other times. These results suggest that conditioned turning responses may be expressed selectively when the intensity of conditioned fear exceeds the threshold for triggering post-encounter defenses, which does not occur unless the US has recently been encountered. Based on these findings, we propose that conditioned turning responses may provide a useful behavioral

index for investigating clinically relevant questions concerning neural substrates that mediate post-encounter defensive strategies.

METHODS

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

Subjects and surgery

Male Long-Evans rats weighing 350–400 g were housed singly and reduced to 85% of ad-lib weight through limited daily feeding. Under deep isoflurane anesthesia, all but two rats (see below) were implanted with a pair of very thin insulated stainless steel wires (75 μ m diameter) threaded into the skin of each eyelid for delivering the mild periorbital shock US. Rats in the experimental groups were implanted with a pair of 26 gauge microinjector guide cannulae (Plastics One, Roanoke, VA) targeted bilaterally in the lateral nucleus of the amygdala (3.0 mm posterior, 5.3 mm lateral and 8.0 mm ventral to bregma) or PAG (7.8 mm posterior, 0.75 mm lateral and 5.8 mm ventral to bregma). All implants were secured in place with bone cement and anchoring screws. At the conclusion of the surgery, rats were removed from the stereotaxic frame and observed until they fully emerged from anesthesia, then returned to their home cages and allowed to recover for at least 5 days prior to beginning experiments. Two rats (one implanted in the amygdala, the other in PAG) were not implanted with periorbital stimulus wires, and were not removed from the stereotaxic frame at the end of the surgery, but instead were given intracranial infusions (0.4 μ l at a rate of 0.25 μ l/min) of fluorescent muscimol (tagged with Bodipy® TMR-X fluorophore, Invitrogen product #M2400), dissolved at 0.25 mg/ml in sterile 0.9% saline vehicle (this was the same volume, concentration, and rate used for infusions of non-fluorescent muscimol in behavioural experiments, see below). 30 min after the infusion was completed, rats were removed from the stereotaxic frame, euthanized with an intraperitoneal overdose of pentobarbital (100 mg/kg), and transcardially perfused with formalin so that brain tissue could be prepared for histological analysis of muscimol diffusion.

Fear conditioning experiments

After recovery from surgery, rats were pre-exposed for 5 days (15 min/day) to the experimental platform before any fear conditioning sessions were conducted. Throughout pre-exposure and fear conditioning sessions, rats constantly foraged on a 70×70 cm platform for 20 mg purified food pellets (Bioserv, Frenchtown, NJ) dropped from an overhead dispenser at ~30 s intervals, to provide a baseline of motor activity against which stimulus-evoked freezing, movement, and turning behavior could be measured. The CS for fear conditioning was a train of 70 dB white noise pips, each lasting 250 ms, delivered at 1 Hz for 20 s through an overhead speaker. The US was a train of very brief 2.0 mA shock pulses, each lasting 2.0 ms, delivered to the skin above one eyelid at a rate of 6.66 Hz for 2 s. During CS-US pairing trials, the first shock pulse was always delivered 300 ms after the offset of the final (20th) CS pip. The interval between CS onset of successive trials was uniformly random between 180 and 240 s for all testing and training trials. Rats implanted with AMG cannulae were trained drug-free for 7 days prior to their first intracranial infusion, whereas rats implanted with PAG cannulae were trained drug-free for 4 days prior to their first infusion.

Rats in the unpaired control group were trained with explicitly unpaired presentations of the CS and US, by delivering the US exactly halfway between CS onset of successive trials (which were separated by a uniformly random interval of 180 and 240 s, as in paired training). In studies of Pavlovian conditioning, it is usually preferable to randomize the order

of CS and US alone trials in the unpaired controls. But here, the conditioned response under study (CS-evoked turning) was strongly modulated by the recency of US delivery. This made it necessary to preserve the alternating order of CS and US presentations in both the paired and unpaired groups, because presenting several CS alone trials in a row to unpaired rats (which would sometimes occur with a randomized trial order) could diminish the CS-evoked turning response by increasing the separation between the CS and the most recent US, and thus reduce conditioned responding by mechanisms unrelated to associative learning. Explicitly unpaired presentations of the CS and US—as we have used here—can cause the CS to acquire properties of a conditioned inhibitor (Rescorla, 1969), and this potential confound is addressed in the Results section.

Behavioral Scoring

The rat's moment-to-moment position on the platform 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, movement, and turning behavior using software developed in our laboratory. The algorithm for scoring freezing behavior has been described elsewhere (Moita et al., 2003; 2004). The algorithm for scoring movement and turning behavior first performed one iteration of smoothing (5-point adjacent averaging) upon the position data for each of the three colored LED's. The center point of the three LEDs was obtained by averaging their x and y coordinates, and the displacement distance of this center position between each successive video frame gave the rat's linear movement speed. The angles of the axes passing through each pair of tracking LED's (red-green, red-blue, blue-green) was measured with respect to the horizontal axis of the video screen. Two of the angles (red-blue and blue-green) were rotated by the appropriate amount to align them with the third angle (red-green), and the mean of these transformed angular measurements was computed using circular averaging to obtain the rat's directional heading for each video frame (if one of the LEDs was occluded, then only one of the three color axes was used to estimate the directional heading). The change in directional heading angle between each successive video frame gave the rat's angular head-turning velocity.

Muscimol Infusions

Muscimol was dissolved at a concentration of 0.25 mg/ml in sterile 0.9% saline vehicle, and infused intracranially at a rate of 0.25 μ l /min. For both amygdala and PAG infusions, a volume of 0.4 μ l was infused into each hemisphere through 33-gauge injectors. Prior to drug infusion, dummy cannulae (which were in place at all times except during infusions, to prevent clogging of the guide cannulae) were removed and injector cannulae were inserted in their place. After drug infusion, the injectors 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. Throughout the infusion process, the animal was held gently on the experimenter's lap. After the infusion was complete, the rat was returned to its home cage for 15 min to allow time for the drug to take effect before the experiment resumed. At the conclusion of the experiment, rats were euthanized by intraperitoneally injected overdose of pentobarbital (100 mg/kg), and transcardially perfused with formalin so that the brain tissue could be removed and sectioned to verify the placements of the injector tips (results shown in Figures 3A and 3B).

RESULTS

Experiment 1: Characterization of conditioned turning responses

Experimental design—Three groups of rats underwent fear conditioning: 1) an amygdala (AMG) group (n=14) that was bilaterally implanted with intracranial infusion cannulae in

the lateral nucleus of the amygdala, 2) a PAG group (n=10) that was bilaterally implanted with infusion cannulae in the lateral and ventrolateral columns of PAG, and 3) an unpaired (UNP) control group (n=10) which was implanted only with periorbital stimulus wires for US delivery, but not with any infusion cannula. On each day of the experiment following initial pre-exposure sessions (see Methods), rats first received 6 test trials in which the CS was presented alone without the US. Following these test trials (after a standard intertrial interval of 180–240 s), rats in the experimental groups (AMG and PAG) received 16 CS-US pairings, while rats in the UNP group received 16 explicitly unpaired presentations of the CS and US. In the PAG and UNP groups, half of the rats received the US on the left eyelid, and the other half on the right. In the AMG group, the US was delivered to the left eyelid of 8 rats and the right eyelid of 6 rats.

Acquisition of conditioned freezing responses—A standard regimen of 6 test trials followed by 16 training trials was given to all three groups of rats on four consecutive days of acquisition training. Since the AMG and PAG groups were trained identically with CS-US pairings on these four acquisition days, the rats in these two groups were combined into a single group designated as the PAIR group (n=24), which was compared against the UNP group to evaluate the effects of paired versus unpaired CS-US presentations upon the acquisition of condition defensive responses.

Figure 1A plots averaged freezing behavior (measured by the amount of time when the headstage tracking lights were immobile) during the context (CX) and CS periods on each trial across days 1–4 of the experiment. The immobility scores from each day were analyzed using a 2×2×2 ANOVA with stimulus (CX vs. CS) and trial type (test vs. training) as repeated factors, and group (PAIR vs. UNP) as an independent factor. The three-way interaction effect (stimulus × trial type × group) was significant on every day after the first day of training (day 1: $F_{1,32}=2.03$, $p=.17$; day 2: $F_{1,32}=35.3$, $p<.00001$; day 3: $F_{1,32}=27.2$, $p=.00001$; day 4: $F_{1,32}=25.0$, $p=.00002$), so all of the factors appeared to influence freezing behavior in trained rats. Newman-Keuls posthoc comparisons revealed that on every day after the first day, rats in the PAIR group froze more during the CS than the CX during test trials (day 1: $p=.21$; day 2: $p=.0003$; day 3: $p=.06$; day 4: $p=.0006$; note that CS-evoked freezing did not quite reach statistical significance on day 3). By contrast, rats in the UNP group never froze more to the CS than the CX during test trials (day 1: $p=.58$; day 2: $p=.44$; day 3: $p=.75$; day 4: $p=.63$). These results agree well with prior studies showing that when an auditory CS has been paired with an eyelid shock US, the CS elicits conditioned freezing responses when it is later presented alone without the US (Moita et al., 2003; Lee and Kim, 2004; Blair et al., 2005a,b). Thus, based on the expression of freezing behavior during test trials (Figure 1A, shaded regions), it appears that rats in the PAIR but not the UNP group acquired conditioned freezing responses to the CS, as expected.

However, the expression of CS-evoked freezing responses during test trials was abruptly reversed during the training trials that followed the test trials on each day. Instead of freezing more to the CS than the CX, rats in the PAIR group froze more to the CX than the CS during training trials (Figure 1A, unshaded regions). This reversal of behavior was evident in the fact that in the PAIR group, the 2×2 interaction between stimulus (CX vs. CS) and session (test vs. training) was significant on all four days (day 1: $F_{1,23}=7.52$, $p=.01$; day 2: $F_{1,23}=81.6$, $p<.00001$; day 3: $F_{1,23}=38.3$, $p<.00001$; day 4: $F_{1,23}=43.4$, $p<.00001$), and freezing to the CX was significantly greater than to the CS during training sessions on days 2–4 (day 1: $F_{1,9}=3.41$, $p=.08$; day 2: $F_{1,9}=27.6$, $p=.00003$; day 3: $F_{1,9}=16.2$, $p=.04$; day 4: $F_{1,9}=4.37$, $p=.048$).

By contrast, rats in the UNP group did not show interaction effects to indicate reversal of freezing responses to the CX versus CS in the transition from testing to training trials (day 1:

$F_{1,9}=0.00003$, $p=.99$; day 2: $F_{1,9}=1.35$, $p=.25$; day 3: $F_{1,9}=4.7$, $p=.04$; day 4: $F_{1,9}=2.52$, $p=.12$). However, the UNP group did show a tendency for greater freezing to the CX than the CS during training sessions on days 2–4 (day 1: $F_{1,9}<.00001$, $p=.99$; day 2: $F_{1,9}=5.46$, $p=.044$; day 3: $F_{1,9}=9.16$, $p=.014$; day 4: $F_{1,9}=5.05$, $p=.051$). This effect was much smaller in the UNP group than in the PAIR group, and probably occurred for a different reason, as will be explained below.

Transition from freezing to turning responses—To determine why rats behaved differently during testing versus training sessions, we conducted further analyses of their movement behavior. Figure 1B plots the average movement speed of the rats across trials on each day. Since freezing is inversely correlated with movement speed, the graphs in Figure 1B look similar to those in Figure 1A, except that the signs of the measurements are reversed. Movement speeds were analyzed using the same $2 \times 2 \times 2$ ANOVA design described above for the freezing analysis. The three-way interaction between stimulus and trial type was again significant on all but the first day of the experiment (day 1: $F_{1,32}=1.8$, $p=.19$; day 2: $F_{1,32}=25.7$, $p=.00002$; day 3: $F_{1,32}=17.4$, $p=.0002$; day 4: $F_{1,32}=18.0$, $p=.0002$). Newman-Keuls posthoc comparisons showed that after day 1, the PAIR group expressed significantly higher movement speeds during the CS than the CX for training trials (day 1: $p=.86$; day 2: $p=.002$; day 3: $p=.009$; day 4: $p=.01$). Rats in the UNP group also showed higher movement speeds during the CS than the CX, but this difference was not significant on any day (day 1: $p=.34$; day 2: $p=.26$; day 3: $p=.17$; day 4: $p=.25$), which is consistent with results reported above showing that both groups froze more to the CX than CS during training sessions, but the effect was more pronounced in the PAIR group. These results indicate that after acquisition of fear conditioning, rats in the both groups froze more and moved less to the CX during training sessions, but the effect was much larger in the PAIR group, and as will be seen below, the underlying cause for this effect was probably different for the PAIR versus UNP groups.

To examine the underlying causes of these conditioned movement responses, we analyzed the rats' turning behavior by using the video tracking system to compute the angular velocity (in degrees/s) of the rat's head throughout each experimental trial (see Methods). This analysis revealed that rats in the PAIR group (but not the UNP group) expressed turning responses to the CS during training trials. These turning responses were strongly biased in the direction opposite from the eyelid where shock was anticipated, as would be expected if they were a flight response away from the shock. Figure 1C plots the average angular turning velocity across trials on each day. Turning responses were analyzed using the same $2 \times 2 \times 2$ ANOVA design described above for the freezing and movement analyses. Once again, the three-way interaction between stimulus and trial type was significant on every day after the first conditioning day (day 1: $F_{1,32}=2.6$, $p=.12$; day 2: $F_{1,32}=5.1$, $p=.03$; day 3: $F_{1,32}=15.8$, $p=.004$; day 4: $F_{1,32}=16.3$, $p=.003$). Newman-Keuls posthoc comparisons revealed that during test trials, rats in the PAIR group showed no significant difference in turning behavior during the CX versus CS on any day (day 1: $p=1.0$; day 2: $p=.99$; day 3: $p=.95$; day 4: $p=.96$). But during training trials, the PAIR group showed a bias for turning away from the shocked eyelid during the CS (day 1: $p=.002$; day 2: $p=.008$; day 3: $p=.0001$; day 4: $p=.0001$). It is clear in Figure 1C that this turning bias was absent during test trials at the beginning of each day, and then emerged during the first few training trials and persisted throughout the rest of the day's training trials. By contrast, rats in the unpaired group never showed a significant turning bias to the CS versus CX during test trials (day 1: $p=.83$; day 2: $p=.95$; day 3: $p=.83$; day 4: $p=.99$) or training trials (day 1: $p=.67$; day 2: $p=.38$; day 3: $p=.93$; day 4: $p=.82$) on any day of the experiment.

These results indicate that during training sessions, rats in the PAIR group did not freeze less to the CS than CX because they were less afraid during the CS than the CX, but because

they expressed their fear of the CS in a different way: by turning rather than freezing. But if this is true, then why did rats in the UNP group also freeze less to the CS than CX, despite showing no turning behavior at all during the CS? Almost certainly, this was because UNP rats actually were less afraid during the CS than the CX. The UNP rats were trained with explicitly unpaired presentations of the CS and US, and it is well established that this procedure can cause the CS to become a “learned safety” signal that inhibits fear responses (Rescorla, 1969). Learned safety effects are not normally measured during training sessions, because freezing behavior tends to be dominated by responses to the US during training (Rogan et al., 2005). This may account for why inhibition of freezing by the CS during training trials was so weak in the UNP group. Based on prior evidence, inhibition of CX-evoked freezing by the explicitly unpaired CS should have been easier to observe when the CS was presented to UNP rats that had not recently encountered the US, during test trials. But the learned safety effect was absent during test trials in the UNP group, because there was very little CX-evoked freezing for the CS to inhibit during these trials. A likely explanation for this is that in hungry UNP rats that had not recently been shocked, the pellet-chasing task caused movements that occluded the CX-evoked freezing responses that would otherwise have been expressed during test trials.

Induction of turning by the US alone—Turning behavior in the PAIR group was not observed during test trials at the beginning of each day, but emerged only during training trials after delivery of the shock US had resumed on that day. To test whether this transition could be induced by the US alone (without the CS), trained rats ($n=12$) were given 8 presentations of the US alone prior to a test session at the beginning of the day (the first CS alone trial was given 120–240 s after the last US alone trial, the standard intertrial interval). The US alone was delivered either to trained or untrained eyelid in counterbalanced order on different days, so that all rats received the US alone on each eyelid.

Figure 2A shows that when rats were not shocked prior to the test session, they exhibited no turning behavior during the CS alone trials, consistent with data from days 1–4 of the acquisition experiment (see Figure 1C). Confirming this, a 2×6 ANOVA of turning responses revealed no main effect of stimulus (CX vs. CS repeated: $F_{1,55}=0.17$, $p=.69$) or trial (1–6 repeated: $F_{5,55}=0.49$, $p=.78$), and no stimulus-by-trial interaction ($F_{5,55}=0.54$, $p=.75$). Figure 2B shows that when rats received the US alone to the trained eyelid prior to the test session, they exhibited turning responses during the first few CS alone trials, which diminished over the course of the test session. Confirming this, a 2×6 ANOVA revealed a significant main effect of stimulus (CX vs. CS repeated: $F_{1,55}=12.97$, $p=.004$) but not trial (1–6 repeated: $F_{5,55}=1.47$, $p=.21$), and a significant stimulus-by-trial interaction ($F_{5,55}=3.08$, $p=.016$). Figure 2C shows that when rats received the US alone to the untrained eyelid prior to the test session, they behaved exactly as when they had been shocked on the trained eyelid: that is, they exhibit turning responses during the first few CS alone trials, which diminished over the course of the test session. Confirming this, a 2×6 ANOVA revealed a significant main effect of stimulus (CX vs. CS repeated: $F_{1,55}=4.85$, $p=.049$) but not trial (1–6 repeated: $F_{5,55}=1.79$, $p=.12$), and a significant stimulus-by-trial interaction ($F_{5,55}=2.84$, $p=.024$). Importantly, the turning responses observed after the US alone were always directed away from the trained eyelid, even when the unpaired US had been delivered to the untrained eyelid. Hence, the directional orientation of the turning response depended upon which eyelid had received the US during training by CS-US pairings, and not upon which eyelid the unpaired US had recently been delivered to. This implies that the recent shock did not affect the previously learned association between the CS and US, but instead served as a trigger for changing the rat's defensive responding from a purely passive mode (freezing) to a more active mode that included turning behavior.

Experiment 2: Dependence of freezing and turning upon amygdala and PAG

Cannula placements—The PAIR group in Figure 1 consisted of 24 rats, and 14 of these (AMG group) were implanted with amygdala infusion cannula, while 10 (PAG group) were implanted with PAG infusion cannula. In two AMG rats, the periorbital stimulus wires stopped functioning (that is, they ceased delivering current) prior to completion of the infusion series, so these rats were dropped from the infusion experiments, reducing the size of the AMG group to $n=12$. Cannula tip placements for the AMG and PAG groups are shown in Figures 3A and 3B, respectively.

Drug diffusion—Figure 3C shows imaging of fluorescent muscimol infused into two rats (which were not included in the behavioral study, see Methods) at the targeted injection sites in AMG and PAG, to illustrate the extent of drug diffusion away from the injection sites at the concentration (0.25 $\mu\text{g}/\mu\text{l}$) and volume (0.4 μl) used in our experiments. In the AMG group, injectors were targeted at the amygdala's lateral nucleus, but Figure 3A shows that some placements were near enough to the borders of the basal or central nuclei that the drug may have diffused into these areas as well. In the PAG group, cannula placements were concentrated mainly in the lateral and ventral columns (Figure 3B), but back-diffusion of the drug along the cannula track may have inactivated neurons in the dorsal column as well (Figure 3C shows diffusion away from an injector tip in the lateral column). It is also possible that muscimol may have affected the overlying superior colliculus, which is known to play a role in oriented movements, and this could in part account for some of the effects reported below upon turning behavior after PAG infusions. We observed pronounced hemispheric effects following unilateral PAG infusions (see below), so unilateral infusions apparently did not spread significantly into the opposite hemisphere. We have recently shown that acquisition of conditioned freezing is blocked by pre-training infusions of muscimol into PAG—but not into lateral offsite control locations—using the same coordinates, concentration, and volume of the drug as in the present study (Johansen et al., 2010). These prior findings support the likelihood that drug effects observed here were probably not caused by lateral diffusion of muscimol out of PAG into other brain regions.

Experimental procedures—After the rats had been trained over 4–7 days of CS-US pairings (see Methods), they were given muscimol infusions to inactivate their respectively implanted brain regions. Each rat in the AMG and PAG groups received a unilateral infusion of muscimol (0.4 μl per side, 0.25 $\mu\text{g}/\mu\text{l}$) into the hemisphere ipsilateral or contralateral from the shocked eyelid (counterbalanced over rats), followed by a standard experimental session of 6 test trials and 16 training trials. The experiment was then suspended for a three-day recovery period, followed by a drug-free retraining session on the fourth day after the infusion. On the day after retraining, a second unilateral infusion was given into the hemisphere opposite from the prior infusion. The experiment was then suspended for another three-day recovery period, followed by another drug-free retraining session on the fourth day. On the next day, the PAG group received bilateral muscimol infusions, but the AMG group did not (instead, the AMG group underwent testing for the effects of unpaired shock delivery upon turning behavior, with results reported above in Figure 2). Using this repeated measures design, each rat in the AMG and PAG groups received separate unilateral infusions of muscimol into each hemisphere (ipsilateral and contralateral from the trained eyelid), and the PAG group received bilateral infusions as well. We did not examine the effects of bilateral amygdala infusions in the present study.

The effects of muscimol infusions upon freezing and turning behavior were analyzed by performing 2×2 ANOVAs on behavior scores with stimulus (CX vs. CS) and inactivation (pre vs. post) both as repeated factors. Separate ANOVAs were performed to analyze the effects of infusions into each hemisphere (ipsilateral, contralateral, and bilateral). In all

analyses presented below, “ipsilateral” and “contralateral” infusion hemispheres are defined with respect to the trained eyelid for each individual rat (for example, ipsilateral infusions were in the left hemisphere for rats trained on the left eyelid, and the right hemisphere for rats trained on the right eyelid, and vice versa for contralateral infusions). For each ANOVA, the “pre” level of the inactivation factor was always comprised from scores obtained during the experimental session that was conducted on the drug-free day immediately prior to the inactivation analyzed by that ANOVA. All posthoc comparisons were performed using the Newman-Keuls test.

Effects of amygdala inactivation on conditioned freezing responses—The effects of infusions upon conditioned freezing behavior were assessed by analyzing behavioral data only from test (CS alone) trials, since CS-evoked freezing was most prominent during these trials (see Figure 1A). Figure 4A summarizes the effects of amygdala inactivation upon freezing behavior during the CX and CS periods of the test trials. The left graph shows that freezing behavior was not affected by inactivation of the amygdala ipsilateral to the US. Confirming this, the 2×2 ANOVA revealed a main effect of stimulus ($F_{1,11}=14.67$, $p=.003$) indicating that rats generally froze more to the CS than CX, and a posthoc comparison detected no significant change in CS-evoked freezing after the infusion ($p=.61$). By contrast, the right graph in Figure 4A shows that contralateral amygdala inactivation significantly reduced CS-evoked freezing responses. The 2×2 ANOVA still exhibited a main effect of stimulus ($F_{1,11}=9.96$, $p=.009$) because of CS-evoked freezing prior to the infusion, but a posthoc comparison revealed that freezing to the CS was significantly reduced after the infusion ($p=.036$). This pattern of results replicates our findings from a previous study, in which it was shown that when rats were trained to fear an auditory CS by pairing it with a unilateral US (as in the present study), expression of CS-evoked freezing was impaired by pre-test inactivation of the amygdala contralateral but not ipsilateral from the US (Blair et al., 2005a).

Effects of PAG inactivation on conditioned freezing responses—Figure 4B summarizes the effects of PAG inactivation upon freezing behavior during the CX and CS periods of the test trials. The leftmost graph shows that ipsilateral PAG inactivation did not significantly affect freezing. The 2×2 ANOVA revealed a main effect of stimulus ($F_{1,9}=13.7$, $p=.005$) indicating that freezing responses were evoked by the CS, and although CS-evoked freezing was slightly reduced after ipsilateral PAG inactivation, this reduction did not reach significance in a posthoc comparison ($p=.12$). The middle graph of Figure 4B shows that after contralateral PAG inactivation, freezing levels tended to increase. There was once again a main effect of stimulus ($F_{1,9}=52.8$, $p=.00005$) to indicate the presence of CS-evoked freezing, but the main effect of drug also approached significance ($F_{1,9}=4.16$, $p=.07$), and posthoc comparisons revealed significantly increased freezing to both the CX ($p=.0003$) and CS ($p=.009$). The rightmost graph in Figure 4B shows that CS-evoked freezing was reduced after bilateral PAG inactivation. Once again there was a main effect of stimulus ($F_{1,9}=14.8$, $p=.004$) to indicate the presence of CS-evoked freezing before inactivation, and CS-evoked freezing was reduced after the infusion ($p=.057$); although this reduction in freezing did not quite reach the .05 level for statistical significance, it is consistent with many prior studies showing that disruptions of PAG impair the expression of freezing behavior (Liebman et al., 1970; LeDoux et al., 1988; Borszcz et al., 1989; Fanselow, 1991; Kim et al., 1993; Johansen et al., 2010).

Effects of amygdala inactivation on conditioned turning responses—Figure 5A summarizes the effects of amygdala inactivation upon turning responses. The left graph shows that ipsilateral amygdala inactivation had no effect upon the rats' turning responses. The 2×2 ANOVA revealed a main effect of stimulus ($F_{1,11}=25.0$, $p=.0004$), reflecting that

fact that rats turned away from the trained eyelid during the CS but not during the CX. Posthoc comparisons showed that this CS-evoked turning bias was present both before ($p=.0006$) and after ($p=.001$) inactivation of the ipsilateral amygdala. There was no main effect of drug ($F_{1,11}=1.71$, $p=.22$) and no stimulus-by-drug interaction ($F_{1,11}=0.27$, $p=.61$). Although CS-evoked turning was reduced slightly after the infusion, this reduction was not significant ($p=.24$), and turning behavior during the CX also remained unchanged ($p=.63$).

The right graph shows that contralateral amygdala inactivation completely abolished CS-evoked turning behavior. Confirming this, the 2×2 ANOVA revealed a main effect of both stimulus ($F_{1,11}=10.1$, $p=.009$) and drug ($F_{1,11}=12.6$, $p=.005$), as well as a stimulus-by-drug interaction ($F_{1,11}=12.3$, $p=.005$). CS-evoked turning was dramatically reduced after the infusion ($p=.003$), while turning behavior during the CX (which was absent to begin with) remained unchanged ($p=.9$). Posthoc comparisons also showed that rats turned away from the trained eyelid significantly more during the CS than the CX before inactivation ($p=.003$), but not after the inactivation ($p=.89$). These results indicate that much like conditioned freezing responses, conditioned turning responses also appear to depend preferentially upon the amygdala contralateral from the trained eyelid in our fear conditioning task.

Effects of PAG inactivation on conditioned turning responses—Figure 5B summarizes the effects of PAG inactivation upon turning behavior. The leftmost graph shows that ipsilateral PAG inactivation significantly altered the rats' turning responses. The 2×2 ANOVA revealed a main effect of stimulus ($F_{1,9}=12.0$, $p=.007$), reflecting that fact that rats turned away from the trained eyelid significantly more during the CS than the CX. Although posthoc comparisons showed that the CS-evoked turning bias was present both before ($p=.003$) and after ($p=.02$) inactivation of the ipsilateral PAG, there was also a main effect of drug ($F_{1,9}=7.33$, $p=.02$) with no stimulus-by-drug interaction ($F_{1,9}=0.86$, $p=.38$). These effects reflected the fact that compared to the pre-inactivation baseline, ipsilateral PAG inactivation caused turning responses to shift toward the trained eyelid by a similar amount during both the CX ($p=.04$) and CS ($p=.005$).

The middle graph in Figure 5B shows that contralateral PAG inactivation also altered the rats' turning behavior, but in a different way from ipsilateral PAG inactivation. The main effects of stimulus ($F_{1,9}=3.55$, $p=.09$) and drug ($F_{1,9}=4.31$, $p=.07$) no longer reached significance (although both showed trends at $p<.1$), whereas the interaction between stimulus and drug was highly significant ($F_{1,9}=31.54$, $p=.0003$). This pattern reflected the fact that rats once again turned away from the trained eyelid significantly more during the CS than the CX before inactivation ($p=.001$), but after inactivation the direction of the turning bias was reversed, so that rats now showed a trend to turn away from the trained eyelid more during the CX than the CS ($p=.06$). This reversal occurred because contralateral PAG inactivation caused the rats to stop turning during the CS ($p=.001$; this may also be regarded as a shift of the turning direction toward the trained eyelid), while at the same time causing a trend for the turning direction to shift away from the trained eyelid during the CX ($p=.06$), despite a baseline of no CX turning at all prior to inactivation.

The rightmost graph in Figure 5B shows that the effects of bilateral PAG inactivation were similar to the effects of contralateral inactivation. The main effects of stimulus ($F_{1,9}=1.11$, $p=.32$) and drug ($F_{1,9}=1.27$, $p=.29$) were not significant, but the interaction between stimulus and drug was significant ($F_{1,9}=18.99$, $p=.002$). Rats turned away from the trained eyelid significantly more during the CS than the CX before inactivation ($p=.006$), but this turning bias was eliminated after inactivation. Bilateral PAG inactivation abolished turning behavior during the CS ($p=.006$) while having no effect on turning during the CX ($p=.16$), which was absent to begin with. After bilateral PAG inactivation, rats showed no significant difference in turning responses to the CX versus CS ($p=.18$).

Persistent turning after unilateral PAG inactivation—Unilateral PAG inactivation induced persistent turning during the CX period of training sessions, which was always directed toward the inactivated hemisphere (that is, towards the side of US delivery for IPSI inactivations, and away from the side of US delivery during CONTRA inactivations). To further investigate this, we analyzed turning responses during test trials that were given after PAG inactivation but before the US had been delivered.

Figure 5C summarizes the effects of PAG inactivation upon turning responses during test sessions. Uninfused rats (“PRE”) never exhibited turning responses to the CX or CS during test trials, in agreement with findings reported above (see Figure 1C). The leftmost graph in Figure 5C shows that ipsilateral PAG inactivation induced persistent turning toward the side of the infusion (or equivalently, toward the trained eyelid) during both the CX and CS. Confirming this, the 2×2 ANOVA revealed no main effect of stimulus ($F_{1,9}=1.09$, $p=.32$) but a significant main effect of drug ($F_{1,9}=19.0$, $p=.002$), with posthoc comparisons indicating a shift of turning responses toward the inactivated hemisphere during both the CX ($p=.02$) and CS ($p=.001$). The middle graph in Figure 5C shows that contralateral PAG inactivation also induced persistent turning, but this time in the direction away from the trained eyelid (which was once again toward the inactivated hemisphere, as in the case of ipsilateral inactivation). Confirming this, the 2×2 ANOVA revealed no main effect of stimulus ($F_{1,9}=0.76$, $p=.4$) but a significant main effect of drug ($F_{1,9}=10.6$, $p=.01$), and posthoc comparisons revealed that PAG inactivation caused turning responses to shift toward the inactivated hemisphere during both the CX ($p=.002$) and CS ($p=.001$). The right most graph in Figure 5C shows that unlike unilateral inactivations, bilateral PAG inactivation did not induce persistent turning during test sessions. Confirming this, the 2×2 ANOVA revealed no main effect of stimulus ($F_{1,9}=0.84$, $p=.38$) or drug ($F_{1,9}=0.29$, $p=.6$) for bilateral PAG inactivation.

In sum, our findings indicate that unilateral (but not bilateral) inactivation of PAG induced persistent turning toward the inactivated hemisphere. This agrees with prior data showing that unilateral inhibition of PAG and surrounding areas can produce turning oriented toward the inactivated side (Geula and Asdourian, 1984). This drug-induced turning behavior complicates our ability to interpret the effects of PAG inactivation upon CS-evoked turning responses, but we shall argue in the Discussion section that the pattern of results observed here is consistent with the possibility that PAG plays a primary role in the expression of CS-evoked turning responses following post-encounter potentiation of conditioned fear.

Effects of muscimol on US processing—Figure 5 showed that conditioned turning responses could be impaired by inactivation of either the amygdala or PAG, especially on the side contralateral (but not ipsilateral) from the trained eyelid. There are two possible ways that inactivation might have produced this impairment of turning behavior. First, inactivation may have shut down neural pathways through the amygdala and PAG that directly mediate expression of CS-evoked turning responses. However, since CS-evoked turning responses do not occur unless rats have recently encountered the US (see Figure 1C), a second possibility is that inactivation may have interfered with nociceptive pathways that mediate sensory processing of the US, and thereby prevented the US from inducing the turning response. These two explanations are not mutually exclusive, since the amygdala and PAG both participate in defensive behavior as well as nociception (Bandler and DePaulis, 1991; Davis, 1992; Keay et al., 1997; Jordan, 1998; Guariu and Bernard, 2004; Neugebauer, 2006).

To investigate how US processing was affected by muscimol, we examined unconditioned movement responses evoked by the US before and after inactivations of amygdala and PAG. Figure 6 shows average movement speeds during US delivery before and after muscimol

infusions. It was found that inactivation of the amygdala ipsilateral to the trained eyelid did not affect US-evoked head movements (Figure 6A, left graph), but inactivation of the contralateral amygdala attenuated US-evoked head movements (Figure 6B, right graph). Confirming this, a 2×2 ANOVA of movements speeds with hemisphere (ipsilateral vs. contralateral) and amygdala inactivation (pre vs. post) as repeated factors yielded main effects of hemisphere ($F_{1,11}=16.8$, $p=.002$) and inactivation ($F_{1,11}=38.8$, $p=.00006$), as well as a significant interaction between hemisphere and inactivation ($F_{1,11}=9.29$, $p=.01$). Posthoc comparisons indicated that the average speed of head movements was unchanged after ipsilateral amygdala inactivation ($p=.14$), but significantly lower after than before contralateral amygdala inactivation ($p=.0007$). US-evoked responses after contralateral inactivation were also lower than after ($p=.002$) or before ($p=.0006$) ipsilateral inactivation. In a prior study, we showed that the periorbital shock US elicits unconditioned head movements from rats, and that these movements are attenuated by bilateral lesions or inactivation of the amygdala (Blair et al., 2005b). Our present findings suggest that the contralateral (but not ipsilateral) amygdala may be the primary contributor to nociceptive processing of the US, which in turn may partly account for the data above showing that the US was no longer able to induce post-encounter potentiation of fear responses after contralateral amygdala inactivation (Figure 5A, right graph).

Figure 6B shows average movement speeds during US delivery before and after muscimol infusions into PAG. Inactivation of the PAG ipsilateral to the trained eyelid did not affect US-evoked head movements (Figure 6A, left graph), but inactivation of the contralateral PAG attenuated US-evoked head movements (Figure 6B, middle graph), as did bilateral PAG inactivation (Figure 6B, right graph). Confirming this, a 3×2 ANOVA of movements speeds with hemisphere (ipsilateral vs. contralateral vs. bilateral) and PAG inactivation (pre vs. post) as repeated factors yielded main effects of hemisphere ($F_{2,18}=5.9$, $p=.01$) and inactivation ($F_{1,18}=136.5$, $p<.00001$), but no interaction between hemisphere and inactivation ($F_{2,18}=2.08$, $p=.15$). Posthoc comparisons indicated that the average speed of head movements was only trending toward reduction after ipsilateral PAG inactivation ($p=.16$), but significantly reduced after contralateral ($p=.0009$) or bilateral ($p=.001$) inactivation of PAG. Hence, it appears that the contralateral (but not ipsilateral) PAG may play a role in nociceptive processing of the US, and this might partially explain why post-encounter potentiation of conditioned fear does not occur after contralateral or bilateral inactivation of PAG.

DISCUSSION

In studies presented here, rats were trained to fear an auditory CS by pairing it with a mild electric shock US delivered to one eyelid, as in prior studies from our laboratory (Moita et al. 2003, 2004; Blair et al. 2005a,b; Tarpley et al., 2009; Johansen et al., 2010). After training, the CS elicited conditioned freezing responses when rats that had not recently encountered the US, but when the same CS was presented to trained rats that had recently encountered the US, it elicited turning in circles away from the trained eyelid where US delivery was anticipated to occur. The CS never elicited such turning responses from rats that were given unpaired presentations of the CS and US, regardless of whether they had recently encountered the US. Pharmacological inactivation experiments revealed that CS-evoked freezing and turning responses were both dependent upon lateralized circuits in the amygdala and PAG, which are known to mediate acquisition and expression of associative fear conditioning (for review, see Fanselow, 1991; Davis, 1992; Maren, 1998; LeDoux, 2000). These findings indicate that fear conditioned rats can express different defensive responses (residing at different points along the predatory imminence continuum) to the same CS, depending upon their recent history of aversive encounters.

Turning as a post-encounter defensive response

When trained rats had not recently encountered a shock, they were presumably in a pre-encounter state of low predatory imminence. Hence, they engaged in pellet-chasing behavior while the CS was not being presented (this is reflected by high movement and low turning scores during the CX period of test trials for paired rats in Figures 1B and 1C). It should be noted that the platform CX may have acquired some association with the shock US via background contextual fear conditioning (since the US was always delivered while rats chased food pellets on the platform). But when rats had not recently been shocked, such context conditioning was apparently not strong enough for the CX to elicit reliable freezing or turning responses by overcoming the rats' competing motivation to chase pellets on the platform. Hence, in rats that had not recently been shocked (that is, during test trials), the CX period appeared to be a time of low predatory imminence during which rats engaged in goal-seeking behavior (that is, pellet chasing). When the CS was presented to trained rats that had not recently been shocked (during test trials at the beginning of each day), they exhibited freezing but not turning responses (reflected by high freezing and low turning scores during the CS on test trials for paired rats in Figures 1A and 1C). Hence, rats that had not recently been shocked may have perceived the CS as a “circa-strike” threat—analogueous to a distant predator that has not yet detected the rat's presence—for which the ethologically programmed response was freezing to avoid detection.

When trained rats had recently encountered a shock, their behavior during both the CX and CS became “shifted to the right” along the predatory imminence continuum. Behavior during the CX after the shock resembled that during the CS prior to the shock: high freezing and low turning scores (see CX data from training trials for paired rats in Figures 1A and 1C). This emergence of CX-evoked freezing could either reflect post-shock freezing elicited by the US, or a latent CX-US association that was too weak to overcome the pellet-chasing drive before shock delivery, but was unmasked after recent encounters with the shock. Consistent with the second possibility, behavior during the CS after the shock was characterized by the emergence of conditioned turning, directed away from the trained eyelid (as indicated by the high turning and moving scores—along with low freezing scores—during training trials for paired rats in Figures 1A–C). Hence, rats that had recently been shocked may have perceived the CS as a “post-encounter” threat—analogueous to a predator that has already detected the rat's presence and recently mounted an attack—for which the ethologically programmed response was flight to avoid/escape further attack. Supporting this interpretation, CS-evoked turning responses exhibited two hallmark characteristics of a post-encounter flight response: 1) turning was expressed preferentially when the CS was presented after a recent encounter with an aversive US (analogueous to recent injury or predatory attack), and 2) turning was oriented in the direction away from the site of anticipated US delivery, moving the rat away from the source of danger.

Neural substrates for fear conditioning and defensive behavior

The perception of threat is often triggered by cues in the environment which signal the presence of danger (such as a fear-conditioned CS), and the amygdala is thought to play a key role in attaching motivational valence to such cues, which allows the organism to accurately perceive the present level of threat in its environment (Weiskrantz, 1956; Davis, 1992; Davis and Whalen, 2001; LeDoux, 2000; Blair et al., 2005b; Seymour & Dolan, 2008). Output from the amygdala is relayed to the PAG (Rizvi et al., 1991), which is one of the low-level brain structures that is critically involved in coordinating the performance of defensive behaviors in response to threats (Chaurand et al., 1972; LeDoux et al., 1988; Bandler & DePaulis, 1991; Fanselow, 1991; DePaulis et al., 1992; Behbehani, 1995; Jordan, 1998; Mobbs et al., 2007, 2009). If we regard the predatory imminence continuum as an innate “defense policy” which maps different threat states onto particular strategies for

evasive action, then it is reasonable to speculate that the current threat level may be signaled by the amygdala, and then relayed (either directly or indirectly) to the PAG, where it is converted into a defensive response that is appropriate for the current threat. Our present findings are consistent with this point of view, and suggest new avenues for future research to investigate how different levels of threat are encoded and converted into specific behavioral responses by the brain's fear system.

Role of the amygdala in CS versus US processing—It is believed 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, 2005). Supporting this view, it has been shown that the amygdala plays an important role in many conditioned defensive responses, including freezing, potentiated startle, autonomic changes in heart rate and blood pressure, and conditioned analgesia (LeDoux et al., 1988; Davis, 1992; Kapp et al., 1992; Helmstetter, 1992; Choi et al., 2001).

In the present study, we observed that two different conditioned fear responses—freezing and turning—were both similarly dependent upon the amygdala (Figures 4 and 5), especially the hemisphere contralateral from the US (see below). This suggests that both conditioned responses depended upon the same memory of the CS-US association, which was stored primarily in one hemisphere of the amygdala. However, since conditioned turning responses were only elicited by the CS after recent encounters with shock, inactivation of the contralateral amygdala may have impaired turning responses not only by blocking recall of the CS-US association, but also by interfering with the ability of the shock to induce turning behavior. Supporting this possibility, we observed here that unilateral inactivation of the amygdala impaired movement responses evoked by a US delivered to the eyelid contralateral from the inactivation (Figure 6). Hence, amygdala inactivation may have impaired CS-evoked turning responses in two different ways: by blocking recall of the CS-US association, and by also attenuating the aversiveness of the shocks upon which turning behavior depended (see Blair et al., 2005b). A good way to further test the role of the amygdala in mediating associative recall versus shock aversiveness would be to inactivate the amygdala ipsilateral to the trained eyelid (which should not impair associative recall) prior to delivering unpaired shocks to the untrained eyelid (opposite from the inactivated hemisphere). We predict that the CS would only elicit freezing but not turning responses in this case, since the memory for the CS-US association would remain intact in the hemisphere contralateral from the trained eyelid (driving the freezing response), but the unpaired shocks would no longer be able to induce turning because their aversiveness would be attenuated by inactivation of the amygdala contralateral from the shock.

Lateralized processing of aversive stimuli by the amygdala—We have previously shown that disruption of the amygdala contralateral (but not ipsilateral) from an eyelid shock US impairs conditioned freezing responses to a CS that predicts the US (Blair et al, 2005a; Tarpley et al., 2009). Here, we found that CS-evoked turning responses and US-evoked reflex movements were also dependent upon the amygdala contralateral from the US. Taken together, these findings suggest that aversive stimulation from the left eyelid may be processed by the right amygdala, and vice versa. However, there is also a body of evidence which suggests that aversive stimuli are processed mainly in the right amygdala rather than the left, regardless of their source (LaLumiere and McGaugh, 1995; Canli et al. 1998; Funayama et al., 2001; Baker and Kim, 2004). For example, it has been shown in rodents that a nociceptive stimulus delivered to either side of the body (left or right) activates chemical and physiological responses primarily in the right rather than the left amygdala (Ji and Neugebauer, 2009; Carasquillo and Gereau, 2008). But these studies examined amygdala responses to chronic inflammatory pain in the limbs, which is relayed to the

amygdala via the anterolateral spinal pathway. By contrast, the eyelid shock US in our studies was an acute aversive stimulus, relayed to the amygdala via trigeminal pathways (see below). Different types of nociceptive stimuli (such as acute versus chronic stimuli, or spinal versus trigeminal stimuli) can elicit a broad variety of different defensive behaviors, and it is possible that lateralization of aversive stimulus processing in the amygdala may depend upon what behaviors are elicited by a particular aversive stimulus.

The turning response we have characterized here is a highly lateralized response, directed toward one side of the body and away from the other. The fact that this turning response depends upon the amygdala contralateral from aversive stimulation (and therefore, ipsiversive to the direction of turning) might provide a clue to the underlying functional reasons why conditioned fear of a unilateral eyelid shock US is processed preferentially in one amygdala hemisphere. Nociceptive signals from the eyelid enter the brain through the ipsilateral trigeminal nucleus of the medullary dorsal horn, which sends weak projections directly to the basal and central nuclei of the contralateral amygdala, and strong (predominantly contralateral) projections to the posterior intralaminar thalamus (Cliffer et al., 1991; Guariou and Bernard, 2004), which in turn sends uncrossed projections to several amygdala subnuclei (LeDoux et al., 1987; 1990). Projections from the central nucleus of the amygdala to PAG are ipsilaterally biased (Rizvi et al., 1991), so outputs from each amygdala hemisphere may preferentially be relayed to PAG on the same side of the brain to drive defensive responses in the proper direction (see below).

Role of the PAG in mediating defensive responses—Disruption of PAG has been shown to impair a variety of defensive responses to threatening stimuli (Liebman et al., 1970; LeDoux et al., 1988; Borszcz et al., 1989; Fanselow, 1991; Kim et al., 1993; Helmstetter & Tershner, 1994; Johansen et al., 2010). In addition, electrical or pharmacological stimulation of PAG can elicit “fight-or-flight” behaviors in the absence of any threatening stimulus, indicating that neural activity in PAG is sufficient for expressing defensive behaviors (DiScala et al., 1984; Bandler & DePaulis, 1991; DePaulis et al., 1992; Keay & Bandler, 2001). Recent evidence from human imaging studies shows that blood oxygen levels in PAG are increased during close encounters with threatening stimuli, suggesting that metabolic activity in PAG is correlated with post-encounter defensive responding (Mobbs et al., 2007, 2009). These findings support the view that expression of conditioned fear responses may depend upon projections from the amygdala, where memories of the CS-US association are stored, to the PAG, which reads out these memories to drive the expression of various conditioned defensive behaviors (LeDoux et al., 1988; Fanselow, 1991; 1994; Zhao and Davis, 2004). It has also been proposed that PAG might be an additional site of associative plasticity (outside of the amygdala) where components of the CS-US association might be stored (Helmstetter et al., 2008). Evidence suggests that the ventral PAG (vPAG) drives passive defensive behaviors such as freezing, whereas the dorsal PAG (dPAG) drives active defensive behaviors, such as flight (DePaulis et al., 1992; De Oca et al., 1998; Kim et al., 1993; Leman et al., 2003; Vianna et al., 2001; Fanselow, 1991). In the present experiments, muscimol infusions were targeted non-specifically at both the lateral and ventral columns of PAG (see Figure 3B), but upward diffusion along the cannula tracks probably led to inactivation of overlying dPAG as well, and possibly the superior colliculus (Figure 3C). PAG inactivation impaired both freezing and turning responses, as would be expected after inactivation of both the vPAG and dPAG.

Lateralized control of defensive responding by PAG—In our experiments, unilateral inactivation of PAG induced persistent turning behavior towards the inactivated hemisphere (Figure 5), and this finding is consistent with prior evidence showing that ipsiversive turning can be induced by unilateral PAG inactivation (Geula and Asdourian, 1984), as well as evidence for the opposite effect that unilateral excitation of PAG can

produce contraversive turning behavior (DePaulis et al., 1992). Based on this pattern of results, it is tempting to conclude that the balance of excitation within the two PAG hemispheres might play a role in “steering” defensive responses in the proper direction to move the animal away from the source of danger. Thus, when the rat experiences or expects delivery of an aversive US on one side of the body, the amygdala may become activated in the contralateral hemisphere, and send outputs to PAG that suppress activity in that hemisphere. This may produce turning behavior towards the side of the activated amygdala and suppressed PAG, and thus, away from the site of US delivery. The superior colliculus overlying PAG may also help to determine the direction of the flight response, since this area is interconnected with PAG (Mantyh, 1982) and plays a role in determining the directional orientation of defensive behaviors (Geula and Asdourian, 1984).

Inactivation of PAG contralateral to the trained eyelid impaired turning (Figure 5B), but spared and may even have enhanced freezing (Figure 4B), perhaps by impairing the competing turning response. Inactivation of PAG ipsilateral to the trained eyelid also spared freezing, but bilateral PAG inactivations impaired freezing (Figure 4B), implying that our muscimol infusions did affect vPAG columns involved in freezing. The tendency for bilateral but not unilateral PAG infusions to impair freezing suggests that both PAG hemispheres may have contributed to the freezing response, even though freezing depends preferentially upon the contralateral amygdala in our task (Figure 4A; Blair et al., 2005a).

Inactivation of PAG ipsilateral to the trained eyelid had an indeterminate effect on turning, because of persistent turning toward the trained eyelid that was induced by the infusion. Even though CS-evoked turning behavior appeared to be absent during training sessions after ipsilateral PAG inactivation (Figure 5B, leftmost graph), this may not have been the case. Instead, CS-evoked turning away from the trained eyelid may have been spared after the inactivation, but masked (or cancelled out) by persistent turning in the opposing direction, resulting in almost no net CS-evoked turning behavior. That is, the rats may have been struggling during the CS to turn “upstream” against the persistent turning in the wrong direction, and succeeded only in a cessation of turning, rather than a full reversal of the turning direction. However, the lack of CS-evoked turning after ipsilateral PAG inactivation could also have been caused by freezing responses that interrupted persistent turning behavior during the CS but not the CX. The effects of ipsilateral PAG inactivation upon turning behavior were thus difficult to ascertain, but the impairment of turning after contralateral PAG inactivation was unambiguous, and the overall pattern of results is consistent with the interpretation that PAG participates in expression of turning behaviors, in agreement with existing evidence that the PAG plays a role in such behaviors (Depaulis et al., 1992; De Oca et al., 1998; Kim et al., 1993; Leman et al., 2003; Vianna et al., 2001; Fanselow, 1991).

Possible mechanisms for US-triggered transition from passive to active defense—One possible neurobiological mechanism for the US-induced transition from freezing to turning behavior could be a change in the responsiveness of vPAG and dPAG to inputs from the amygdala. When rats have not recently been shocked, CS-evoked activity in the amygdala may trigger vPAG neurons to produce freezing responses, whereas after rats have recently been shocked, the same CS-evoked activity in the amygdala may instead trigger dPAG neurons to produce flight responses. Perhaps vPAG and dPAG might compete with one another for control over defensive responding (see Walker et al., 1997), and shock delivery might alter the balance of this competitive interaction so that prior to shock delivery, competition is biased in favor of the vPAG, but after shock delivery, the bias shifts to favor dPAG. It has been proposed (see Deakin & Graeff, 1991) that defensive behavior might be influenced by neuromodulatory systems which are known to regulate neural activity in PAG (such as opiates, dopamine, and serotonin), and activation of these

neuromodulators by shocks is one possible mechanism by which the responsiveness of vPAG and dPAG to input from the amygdala might be altered to produce changes in defensive behavior.

In addition to the amygdala-PAG pathway, there is also evidence that the medial prefrontal cortex (mPFC) might play a significant role in the expression of conditioned freezing responses (Blum et al., 2006; Corcoran and Quirk, 2007; Burgos-Robles et al., 2009). The mPFC receives input from the amygdala and sends major projections to PAG (McDonald, 1991; Floyd et al., 2000), so modulation of neural activity in mPFC by shocks could be another mechanism by which shock delivery might alter the rat's defensive response strategy. Further studies are warranted to investigate how different brain structures (including amygdala, mPFC, vPAG, and dPAG) and neuromodulators regulate US-triggered transitions between conditioned freezing and turning responses. It will also be important for future studies to examine whether induction of CS-evoked turning by the US can occur in contexts other than the one where the CS-US association was learned, and whether arousing stimuli other than eyelid shocks can induce CS-evoked turning behavior in fear conditioned rats.

Implications for clinical anxiety disorders

The neural circuits that regulate fear expression have been widely studied, and rodent fear conditioning has been adopted as a dominant animal model for investigating the neurobiological basis of clinical anxiety disorders (Davis and Whalen, 2001; Rau et al., 2005; Davis et al., 2006; Milad et al. 2006; Miller & McEwen, 2006; Rauch et al., 2006). It has been proposed that patients with anxiety disorders may suffer from over-acquired fear associations, so that neutral cues which should not predict danger (or only predict a moderate level of danger) instead predict a high level of danger, leading to over-activation of the amygdala and inappropriate defense responses (Davis and Whalen, 2001; Rau et al., 2005; Rauch et al., 2006). In human patients, such inflated fear responses might result from an inability to normally extinguish conditioned fear associations, and this possibility has generated considerable interest in therapies that focus on helping patients with anxiety disorders to extinguish their fear memories (Davis et al., 2006; Milad et al., 2006).

However, the findings reported here emphasize that defensive responses depend not only upon long-lasting associative fear memories that have been acquired in the remote past, but also upon transient encounters with aversive stimuli that have occurred in the recent past. In healthy people, a recent brush with danger may acutely enhance defensive responses to mildly threatening stimuli (to promote escape from recently encountered threats that may still be present in the environment), without causing long-term changes in the aversive valence of such stimuli. But in some patients with anxiety disorders, this normally acute state might become chronic, so that the patient always behaves as if they have recently had a brush with danger (even when they have not), and thus always exhibits exaggerated defensive responding to mildly threatening stimuli. In other words, such patients might always behave as if they are in a "training session" (behaving as though a US has recently been delivered) even when they are actually in a "test session" (because no US has in fact been recently encountered).

Our present findings imply that in rodents, the mode of conditioned fear expression depends strongly upon recent (rather than remote) events (see also Mongeau et al., 2003). This fact highlights the possibility that neurobiological mechanisms underlying chronically heightened defensiveness might be independent from the long-term memory processes that mediate the storage of CS-US associations. Instead, chronically heightened defensiveness might sometimes be caused by dysfunction of short-term memory systems that register the recent occurrence of aversive events, or by chronic activation of hormonal and

neuromodulatory systems that are acutely activated by such aversive events in healthy people. To further investigate how these factors might contribute to anxiety disorders, it will be necessary to identify the neural mechanisms by which encounters with aversive events can acutely modulate the mode of defensive responding. The novel conditioned turning response we have described here may provide a helpful tool for observing and measuring US-triggered transitions from circa-strike to post-encounter defensive responding in fear conditioned rats, and may thus provide a useful animal model for investigating how brain systems that are acutely activated during aversive stimulus encounters might contribute to clinical anxiety disorders.

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Figure 1. Acquisition and state-dependent expression of conditioned freezing and flight responses

Each graph shows averaged behavioral responses over 6 test trials (gray shaded regions) and 16 training trials (unshaded regions) on a single day of the experiment. A) Freezing behavior, measured as the amount of time the headstage tracking lights were immobile during the trial. B) Head movement speed, measured as the mean speed of the headstage lights (in cm/s) during the trial. C) Head turning velocity, measured as the speed of angular rotation (in deg/s) of the headstage lights; positive values indicate turning in the direction away from the shocked eyelid, whereas negative values indicate turning toward the shocked eyelid.

Figure 2. Induction of CS-evoked turning responses by unpaired shocks prior to test
 Each graph shows mean CS-evoked turning scores over 6 trials of a test session (n=12 rats).
 A) Standard test session that was not preceded by shocks. B) Test session preceded by 8 unpaired shocks to the trained side. C) Test session preceded by 8 unpaired shocks to the untrained side.

Figure 3. Cannula placements and drug diffusion

Reconstructed placements of infusion cannula for the AMG (panel A) and PAG (panel B) groups are shown using templates from the atlas of Paxinos and Watson (1997). Hemispheres are labeled ipsilateral (IPSI) versus contralateral (CONTRA) with respect to the trained eyelid for each rat. Panel C shows imaging of fluorescent muscimol (red) against DAPI counterstain (blue) to illustrate diffusion of the drug away from injection sites in amygdala and PAG.

Figure 4. Effects of muscimol infusions on freezing behavior

Each graph shows averaged freezing scores before (PRE) versus after (POST) muscimol infusions into a particular hemisphere (IPSI, CONTRA, or BILAT) of the amygdala (panel A) or PAG (panel B). Symbols above black bars denote significance of the comparison with the adjacent white bar. Symbols above connector lines denote significance of the comparison between bars connected by that line.

Figure 5. Effects of muscimol infusions on turning behavior

Each graph shows averaged turning scores before (PRE) versus after (POST) muscimol infusions into a particular hemisphere (IPSI, CONTRA, or BILAT) of the amygdala (panel A) or PAG (panels B and C). Symbols adjacent to connector lines denote significance of the comparison between dots connected by that line.

Figure 6. Effects of muscimol infusions on US-evoked movement

Each graph shows averaged movement speed during the US before (PRE) versus after (POST) muscimol infusions into a particular hemisphere (IPSI, CONTRA, or BILAT) of the amygdala (panel A) or PAG (panel B). Symbols adjacent to connector lines denote significance of the comparison between bars connected by that line.