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Neural substrates for expectation-modulated fear learning in the amygdala and periaqueductal gray

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A form of aversively motivated learning called fear conditioning occurs when a neutral conditioned stimulus is paired with an aversive unconditioned stimulus (UCS). UCS-evoked depolarization of amygdala neurons may instruct Hebbian plasticity that stores memories of the conditioned stimulus–unconditioned stimulus association, but the origin of UCS inputs to the amygdala is unknown. Theory and evidence suggest that instructive UCS inputs to the amygdala will be inhibited when the UCS is expected, but this has not been found during fear conditioning. We investigated neural pathways that relay information about the UCS to the amygdala by recording neurons in the amygdala and periaqueductal gray (PAG) of rats during fear conditioning. UCS-evoked responses in both amygdala and PAG were inhibited by expectation. Pharmacological inactivation of the PAG attenuated UCS-evoked responses in the amygdala and impaired acquisition of fear conditioning, indicating that PAG may be an important part of the pathway that relays instructive signals to the amygdala.

The amygdala is an important site of neural plasticity, where associative memories are stored during fear conditioning[1–5]. Evidence suggests that storage of fear memories requires Hebbian long-term potentiation at conditioned stimulus input synapses onto neurons in the lateral nucleus of the amygdala[1,2,6–11]. This Hebbian plasticity is thought to be triggered by the UCS, which causes postsynaptic depolarization of lateral nucleus of the amygdala (LAn) neurons in conjunction with presynaptic activation of conditioned stimulus inputs[1,2,5,6]. If so, then afferent pathways that transmit UCS information to the amygdala can be regarded as 'teaching inputs' that instruct associative plasticity at conditioned stimulus input synapses.

A number of studies have attempted to identify the teaching input pathways that convey UCS information to the amygdala during fear learning[12–16], but it remains unclear which neural circuits mediate this function. Behavioral evidence from blocking experiments suggests that fear conditioning may be instructed by UCS signals that are inhibited by expectation, rather than by a simple sensory representation of the UCS[17–21]. Modulation of neural signals by expectation has been seen in many learning systems[22–25], and recent studies have provided neurophysiological evidence that responses of amygdala neurons to aversive (or appetitive) stimuli are also modulated by expectation[26,27]. However, it is not clear whether this occurs during Pavlovian fear conditioning at sites of associative plasticity (such as the LAn) or in brain regions that participate in relaying UCS information to the amygdala.

We investigated how UCS information was processed by neurons in the amygdala and PAG during fear conditioning and examined whether the PAG is part of the UCS pathway. We found that UCS processing was modulated by expectation in both the LAn and PAG. Our results suggest that PAG may be essential for relaying expectancy-modulated UCS information to instruct associative plasticity in the LAn and for regulating fear memory formation.

RESULTS

Single-unit recording in amygdala during fear conditioning

To investigate whether UCS processing in the LAn is modulated by expectation during fear conditioning, we implanted rats ($n = 14$) with recording electrodes targeted at the LAn and with periorbital wires for delivering the shock UCS to the skin above one eyelid. When well-isolated units were encountered in LAn, a pre-conditioning session consisting of six presentations of an auditory conditioned stimulus (CSa) was given to assess baseline responses to context and CSa, followed immediately by a conditioning session consisting of 16 CSa-UCS pairings. After a 1-h delay, conditioned responding to the CSa was assessed during a post-conditioning test session consisting of six presentations of the auditory stimulus alone. Most rats underwent additional recording sessions on subsequent days to further assess UCS-evoked responses of amygdala neurons.

Rats acquired conditioned freezing responses to the CSa during single-unit recording sessions (**Fig. 1**). A 2 × 2 repeated-measures ANOVA of freezing scores revealed a significant interaction ($F_{1,12} = 9.3$, $P = 0.01$) between stimulus condition (context versus CSa) and session (pre versus post conditioning; **Fig. 1a**). The freezing levels that we observed were similar to those observed in prior studies using the same fear conditioning procedure[28], which are lower than in fear conditioning studies using a standard foot shock UCS (Online Methods).

Changes in UCS-evoked responses during conditioning

If UCS processing is modulated by expectation, then UCS-evoked responses should become attenuated during training as rats learn that

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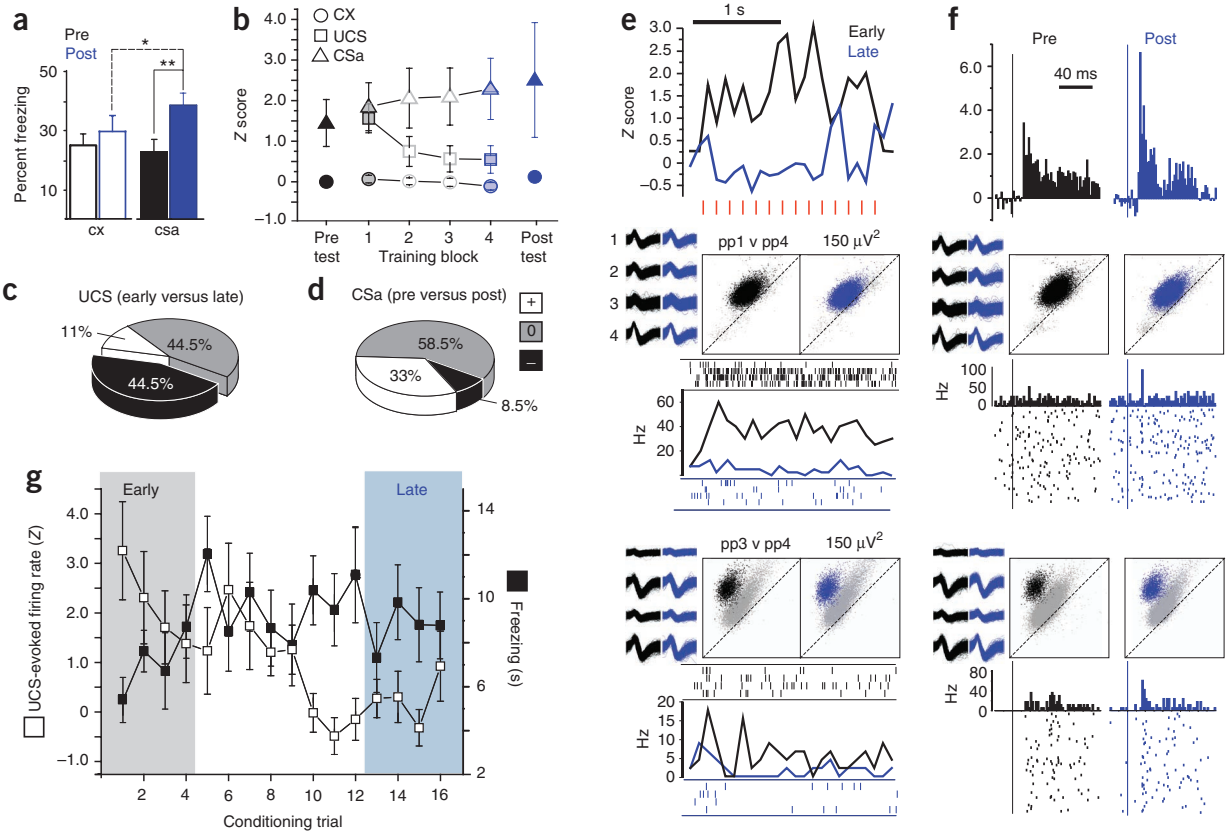

Figure 1 Activity of LAn neurons during acquisition of fear conditioning. (**a**) Freezing behavior during the 20-s context (CX) and CSa periods before (pre) and after (post) fear conditioning (*P = 0.004, **P = 0.0007, Newman-Keuls *post hoc* test). (**b**) Normalized stimulus-evoked responses (y axis) averaged over the population of shock-responsive LAn neurons (n = 27) for each of the four conditioning trial blocks (four trials per block) and for the first four trials of the pre- and post-conditioning test sessions. (**c**) Pie chart showing the percentage of shock-responsive LAn cells that significantly reduced (–), increased (+) or did not change (0) their UCS-evoked responses between the first (early) and last (late) conditioning trial block. (**d**) Pie chart showing the percentage of shock-responsive LAn cells that significantly changed their auditory responses between the pre- and post-conditioning test sessions. (**e**) Top, peristimulus time histogram (PSTH, bin size = 100 ms) showing normalized activity during shock trains (individual shock pulses indicated by red hash marks) for early versus late conditioning trials, averaged over the subpopulation of LAn neurons that significantly reduced their shock-evoked response (n = 12). Bottom, PSTHs and spike rasters showing UCS-evoked responses for two example neurons from different rats, along with waveforms and cluster plots for spikes fired during the session (ppX v ppY above scatter plots denotes channel numbers for peak-to-peak spike voltages plotted on the x and y axes, respectively). (**f**) Top, PSTH (bin size = 2 ms) showing auditory responses (onset of white noise pip indicated by vertical line) during the pre- versus post-conditioning test sessions for the subpopulation of LAn cells shown in **e**. Bottom, auditory responses for the same two example neurons shown in **e**. (**g**) Responses to the UCS (left y axis) on each of the 16 conditioning trials (averaged over the subpopulation of LAn cells that significantly reduced their UCS-responsiveness during conditioning) are graphed alongside average freezing scores (right y axis) during the CSa period on each trial.

the CSa predicts the UCS. To test this, we recorded from a total of 35 well-isolated neurons in the LAn from 11 of the 14 rats during the initial training session (**Supplementary Fig. 1**). Shock-evoked responses were observed in 77% (27 of 35) of the neurons that were recorded during training. Of these 27 shock-responsive neurons, 24 were held throughout the pre- and post-conditioning test sessions as well as the training session. To analyze how the activity of these shock-responsive neurons changed during conditioning, we subdivided the 16 conditioning trials into four consecutive blocks of four trials each (**Fig. 1b**). A 4 × 2 ANOVA revealed a significant interaction ($F_{3,78}$ = 3.64, P = 0.016) between trial block and stimulus. *Post hoc* comparisons revealed that UCS-evoked responses during trial block 1 were significantly greater than during blocks 2–4 (P < 0.0006 for every comparison). The context baseline did not differ significantly between any pair of trial blocks (P > 0.67 for every comparison), indicating that baseline firing rates remained stable throughout the conditioning session. These findings indicate that UCS-evoked responses of LAn neurons decreased during early conditioning trials (block 1) and then remained attenuated throughout the remainder of the session (blocks 2–4).

We analyzed UCS-evoked responses of individual neurons to determine which cells changed their responses to the shock UCS between the first and last conditioning trial blocks (Online Methods). Of the shock-responsive cells, 44.5% (12 of 27) reduced their shock responsiveness over the course of training (**Fig. 1c**,**e**), whereas 11% (3 of 27) increased their shock responsiveness and 44.5% (12 of 27) showed no change. Thus, individual LAn neurons were fourfold more likely to decrease (n = 12) than to increase (n = 3) their response to the shock UCS during the training session and this was a statistically significant bias toward diminution of UCS-evoked responses (two-tailed binomial test, P = 0.035). Analysis of baseline firing rates suggested that diminution of UCS-evoked responding during training did not occur preferentially in principle cells versus interneurons (**Supplementary Fig. 2**).

Conditioned changes in auditory-evoked responses

Previous studies have reported that auditory fear conditioning enhances CSa-evoked responses of LAn neurons at short time latencies after conditioned stimulus onset and these findings have been taken as evidence for conditioning-induced potentiation of input pathways that relay the CSa to the amygdala from the thalamus or cortex[9,10]. To examine this, we compared short-latency CSa-evoked responses from the pre- versus post-conditioning test sessions. Auditory responses were larger after than before conditioning (**Fig. 1f**), but this enhancement did not reach statistical significance in the population of shock-responsive cells (paired t_{23} = 1.46, P = 0.16). However, analysis of individual neurons revealed that short-latency CSa-evoked responses were enhanced in 33% (8 of 24) of the cells, whereas 58.5% (14 of 24) of the cells showed no change in their firing to the CSa after conditioning and 8.5% (2 of 24) of the cells showed a decrease in auditory-evoked responses (**Fig. 1d**). These results are consistent with previous electrophysiological, cellular and molecular evidence indicating that fear conditioning enhances short-latency to the CSa responses in a subset of amygdala neurons, but not in all neurons[7–10,27,29,30].

A 2 × 2 ANOVA compared UCS-evoked responses for cells that exhibited conditioned enhancement of auditory responding (n = 8) versus those that did not (n = 16). There was a significant main effect of trial block (block 1 versus 4, $F_{1,22}$ = 6.35, P = 0.019), but no effect of cell type (CSa enhanced versus non-enhanced, $F_{1,22}$ = 0.12, P = 0.73) and no interaction between trial block and cell type ($F_{1,22}$ = 0.56, P = 0.46). Thus, diminution of UCS-evoked responses in individual cells did not depend on whether the cell showed conditioned enhancement of its auditory responses (see Discussion).

Modulation of UCS processing by the predictive stimulus

Expectation of an aversive stimulus can attenuate the responsiveness of amygdala neurons to that stimulus[27], so UCS-evoked responses of LAn neurons may have decreased during conditioning as rats learned to expect the shock to follow the predictive CSa. Supporting this interpretation, freezing behavior during the CSa increased during early conditioning trials and remained elevated for the remainder of the trials (**Fig. 1g**). For cells that diminished their responses to shock (n = 12), the magnitude of UCS-evoked responses was inversely correlated with CSa-evoked freezing responses across conditioning trials (r_{16} = –0.53; P = 0.03), suggesting that diminution of shock responses was related to the rats' acquired expectation of the shock.

To further investigate whether diminution of shock responses was attributable to a learned expectation of the shock, we conducted additional recording sessions after the initial conditioning session in 9 of the 14 rats. In these later sessions, previously conditioned rats were given 16 presentations of the shock UCS, of which eight were preceded by a previously trained CSa (signaled UCS trials) and eight were not (unsignaled UCS trials), given in random order. In some rats, electrodes were advanced below the LAn and into the basal nucleus during later recording sessions (**Supplementary Fig. 1**). Neurons from both nuclei were combined for our analyses.

A total of 70 well-isolated LAn and basal nucleus neurons were recorded from nine rats during 24 signaled-unsignaled shock sessions that followed conditioning. Significant shock-evoked responses were observed in 45 of 70 (64%) of these neurons and, in this population, responses to the unsignaled UCS were significantly larger than to the signaled UCS (t_{44} = 2.9, P = 0.006). Analysis of individual neuron responses revealed that LAn and basal nucleus neurons were five-fold more likely to respond preferentially to the unsignaled UCS (n = 15) than to the signaled UCS (n = 3; **Fig. 2a–c**), which was a significant bias for preferential responding to the unsignaled UCS (two-tailed binomial test, P = 0.0075). Differential responses of LAn and basal nucleus neurons to predicted versus unpredicted shocks were not attributable to differences in motor responses to the shocks (**Supplementary Fig. 3**).

If fear conditioning is instructed by UCS-evoked depolarization in the amygdala, then the reduction in UCS-evoked responses of LAn and basal nucleus neurons that we observed following a predictive CSa should be accompanied by a reduction in the ability of the UCS to produce fear conditioning. To test this, we conducted a blocking experiment in which all rats first received pre-exposure to the CSa and a visual flashing light conditioned stimulus (CSv) and then eight rats (blocked group) received 16 pairings of the CSa with the eyelid shock UCS and another eight rats (naive group) were not trained. Both groups then received 16 pairings of a compound conditioned stimulus, consisting of the CSa combined with the CSv, and the eyelid shock UCS. The CSv by itself evoked less freezing from blocked than from naive rats 24 h later, as revealed by a significant 2 × 2 interaction effect between stimulus (context versus CSv) and treatment group (blocked versus control; $F_{1,14}$ = 6.1, P = 0.03; **Fig. 2d**). These results suggest that, in the presence of the predictive CSa, both UCS-evoked responses of amygdala neurons and the ability of the UCS to instruct fear conditioning were similarly reduced.

PAG inactivation attenuates responding to shock in amygdala

Blocking of fear conditioning by a predictive conditioned stimulus may be mediated by conditioned analgesia, whereby the conditioned stimulus activates outputs from amygdala to PAG, which in turn inhibits nociception (and thus blocks UCS processing) at the level of the spinal and trigeminal dorsal horn[18–20,31]. If so, then PAG inactivation should prevent antinociception from occurring in the dorsal horn and thereby prevent the conditioned stimulus from inhibiting UCS-evoked responses in amygdala (without affecting responses to the UCS in the absence of the conditioned stimulus). To test this, we inactivated PAG with muscimol (MUS) while responses to signaled and unsignaled shocks were recorded from LAn neurons in previously fear-conditioned rats (**Fig. 3**). A total of 15 well-isolated LAn neurons were recorded from three rats before and after MUS (0.4 µl per side, 0.25 mg ml^{-1}) was microinjected into the PAG (**Supplementary Fig. 4**), of which 13 of 15 (87%) were responsive to the eyelid shock UCS. Of these shock responsive neurons, 7 of 13 (54%) responded preferentially to unsignaled presentations of the UCS (**Fig. 3a,c,d**).

A 2 × 2 ANOVA revealed a significant interaction between inactivation (pre versus post) and shock type (signaled versus unsignaled, $F_{1,12}$ = 9.48, P = 0.009). *Post hoc* comparison tests confirmed that amygdala neurons responded significantly more to unsignaled than to signaled shocks before inactivation (P = 0.0008), but the cells no longer responded differently to predicted versus unpredicted shocks after PAG inactivation (P = 0.86), mainly because amygdala neurons no longer responded to shocks of either type (**Fig. 3a,c**). There was a significant reduction in the population response to both signaled (P = 0.006) and unsignaled (P = 0.0002) shocks after PAG inactivation. However, PAG inactivation did not affect responses to the CSa (paired t_{12} = 1.23, P = 0.24; see **Fig. 3b**) or baseline firing rates (paired t_{12} = 0.96, P = 0.35) of amygdala neurons. These data provide evidence that, in addition to its role as an output structure for mediating conditioned fear responses such as freezing and analgesia, the PAG may also participate in relaying UCS information to the LAn.

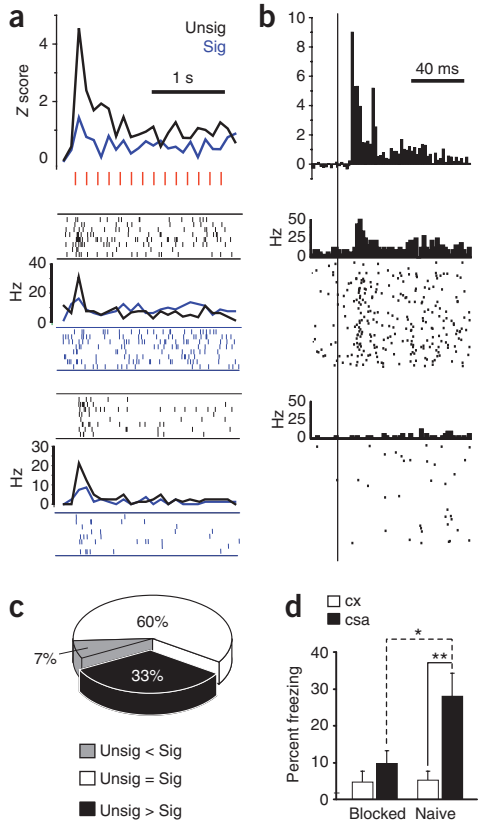

Figure 2 LAn/basal neurons responded more to unsignaled (unsig) than signaled (sig) shocks. (**a**) Top, PSTHs (bin size = 100 ms) showing normalized activity during signaled and unsignaled shock trains (individual shock pulses indicated by red hash marks) for the subpopulation of LAn and basal nucleus neurons ($n = 15$) that responded significantly more to unsignaled than to signaled shocks. Bottom, PSTH and spike rasters showing responses to signaled and unsignaled shocks for two example neurons from different rats. (**b**) Top, PSTH (bin size = 2 ms) showing normalized auditory responses (onset of white noise pip indicated by vertical line) during signaled trials for the subpopulation of LAn and basal nucleus neurons shown in **b**. Bottom, PSTHs and spike rasters showing auditory responses for the same two example neurons shown in **b**. (**c**) Pie chart showing the percentage of shock-responsive LAn and basal nucleus cells that responded significantly more to unsignaled than to signaled shocks (black), significantly more to signaled than to unsignaled shocks (gray) or the same to both types of shock (white). (**d**) Freezing during the final test session of the blocking experiment (*$P = 0.004$, **$P = 0.002$, Newman-Keuls *post hoc* test).

PAG inactivation impairs acquisition of conditioned freezing

If PAG participates in relaying aversive UCS information to the amygdala to instruct associative plasticity, then the acquisition of fear conditioning should require the PAG, and some prior evidence suggests that this is true (Bellgowan, P.S.F., Helmstetter, F.J. and Bailey, D.J. *Soc. Neurosci. Abstr.* **442.12**, 1996). To investigate whether the PAG was necessary for acquisition of fear conditioning, we micro-injected MUS (0.4 µl per side, 0.25 mg ml^{-1}) or vehicle into the PAG before training. Conditioned freezing was assessed during a drug-free test session given 6 d later (**Supplementary Fig. 5**).

Prior to fear conditioning, rats did not exhibit freezing to the context or CSa (**Fig. 4a**). During the post-conditioning test session, vehicle rats ($n = 11$) froze to the CSa, whereas MUS rats ($n = 12$) did not (**Fig. 4b**), as revealed by a significant interaction ($F_{1,21} = 11.180$, $P = 0.003$) between drug treatment (MUS versus vehicle) and stimulus condition (context versus conditioned stimulus). During conditioning, MUS rats exhibited reduced unconditioned reflex responses (head movement) to the shock (**Supplementary Fig. 6**). Impairment of fear learning with PAG infusions was not attributable to MUS spreading into brain regions lateral to the PAG, as another group of control rats that received pre-training MUS into sites lateral to the PAG acquired conditioned freezing responses (**Supplementary Fig. 7**). Impaired fear learning in MUS rats was also not caused by permanent damage to PAG, as impaired rats learned normally when retrained in drug-free conditions (**Supplementary Fig. 8**). We also found (**Supplementary Fig. 9**) that PAG inactivation reduced expression of conditioned freezing in well-trained rats, replicating prior findings[31–34]. Thus, in addition to its known role as an output structure for conditioned fear responses, the PAG also appears to be necessary for the acquisition of fear memory formation.

UCS processing in PAG neurons is modulated by expectation

These findings suggest that the PAG participates in relaying UCS signals to the amygdala. As UCS-evoked responses in the amygdala

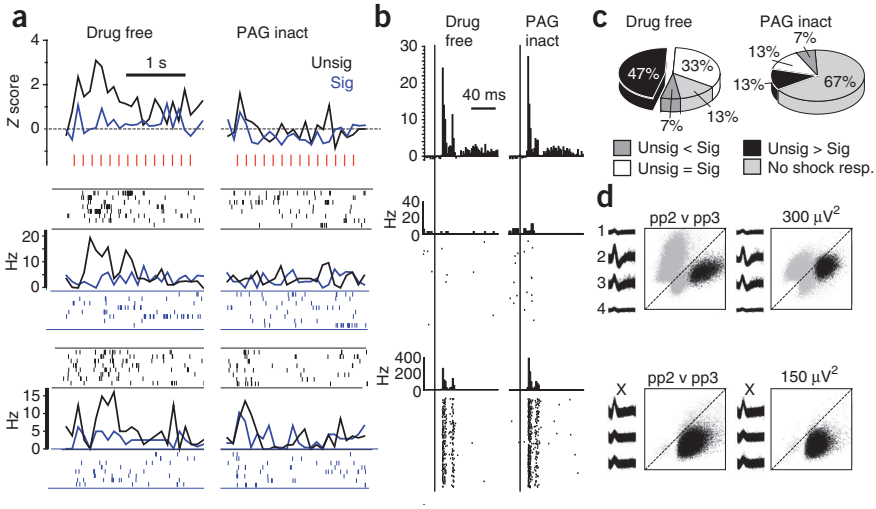

Figure 3 Attenuation of shock-evoked responding in LAn neurons by PAG inactivation. (**a**) Top, PSTHs (bin size = 100 ms) showing normalized activity during signaled and unsignaled shock trains (individual shock pulses indicated by red hash marks) averaged over LAn cells ($n = 7$) that responded significantly more to unsignaled than to signaled shocks. Bottom, PSTHs and spike rasters showing responses for two example neurons from different rats. (**b**) Top, PSTHs (bin size = 2 ms) showing normalized auditory responses during signaled trials before versus after infusions of MUS into PAG for the same neurons shown in **a**. Bottom, PSTHs and spike rasters showing auditory responses for the neurons shown in **a**. (**c**) Pie chart summarizing how UCS-evoked responses of LAn cells that responded to shock before PAG inactivation ($n = 13$) changed after inactivation. (**d**) Spike waveforms and cluster plots for the example cells in **a** before (left) and after (right) PAG inactivation.

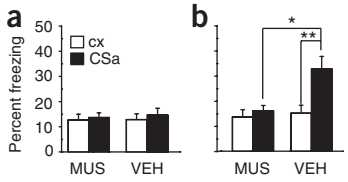

Figure 4 Effects of PAG inactivation on fear conditioning. (**a**) Pre-training freezing levels in rats that subsequently received PAG micro-injections of MUS or vehicle (VEH) before conditioning. (**b**) Freezing during a test session given 6 d after conditioning. $*P = 0.002$, $**P = 0.0002$.

were modulated by expectation and PAG inactivation reduced UCS-evoked responses in lateral nucleus of the amygdala neurons, we next examined whether UCS-evoked responses of PAG neurons were also modulated by expectation. Rats ($n = 13$) were implanted with recording electrodes targeted to pass through the dorsal, lateral and ventral columns of PAG (**Supplementary Fig. 10**) and recording and behavioral procedures were as described above for the amygdala recording experiments, but without pre- and post-conditioning test sessions.

A total of 21 well-isolated PAG neurons were recorded from eight rats during their initial conditioning session and 95% (20 of 21) of these cells were responsive to shocks (**Supplementary Fig. 11**). A 4×2 ANOVA revealed an interaction between stimulus (context versus CSa) and trial block ($F_{3,57} = 5.78$, $P = 0.002$) and *post hoc* comparisons showed that UCS-evoked responses were significantly reduced during blocks 3 ($P = 0.03$) and 4 ($P = 0.003$) compared with block 1 (**Fig. 5a**). The context baseline did not differ significantly between any pair of trial blocks ($P > 0.55$ for every comparison), indicating that baseline firing rates remained stable throughout the conditioning session. These findings indicate that UCS-evoked responses of PAG neurons decreased during conditioning.

To analyze how individual PAG neurons changed their responses to the UCS during conditioning, a statistical comparison was made between each cell's responses during the first versus last trial block. UCS-evoked responses decreased in 50% (10 of 20) of the cells (**Fig. 5b–d**), increased in 15% (3 of 20) of the cells and remained unchanged in 35% (7 of 20) of the cells (**Fig. 5b**). Thus, individual PAG cells were more likely to decrease than to increase their responsiveness to the UCS during conditioning, although this tendency did not reach statistical significance (two-tailed binomial

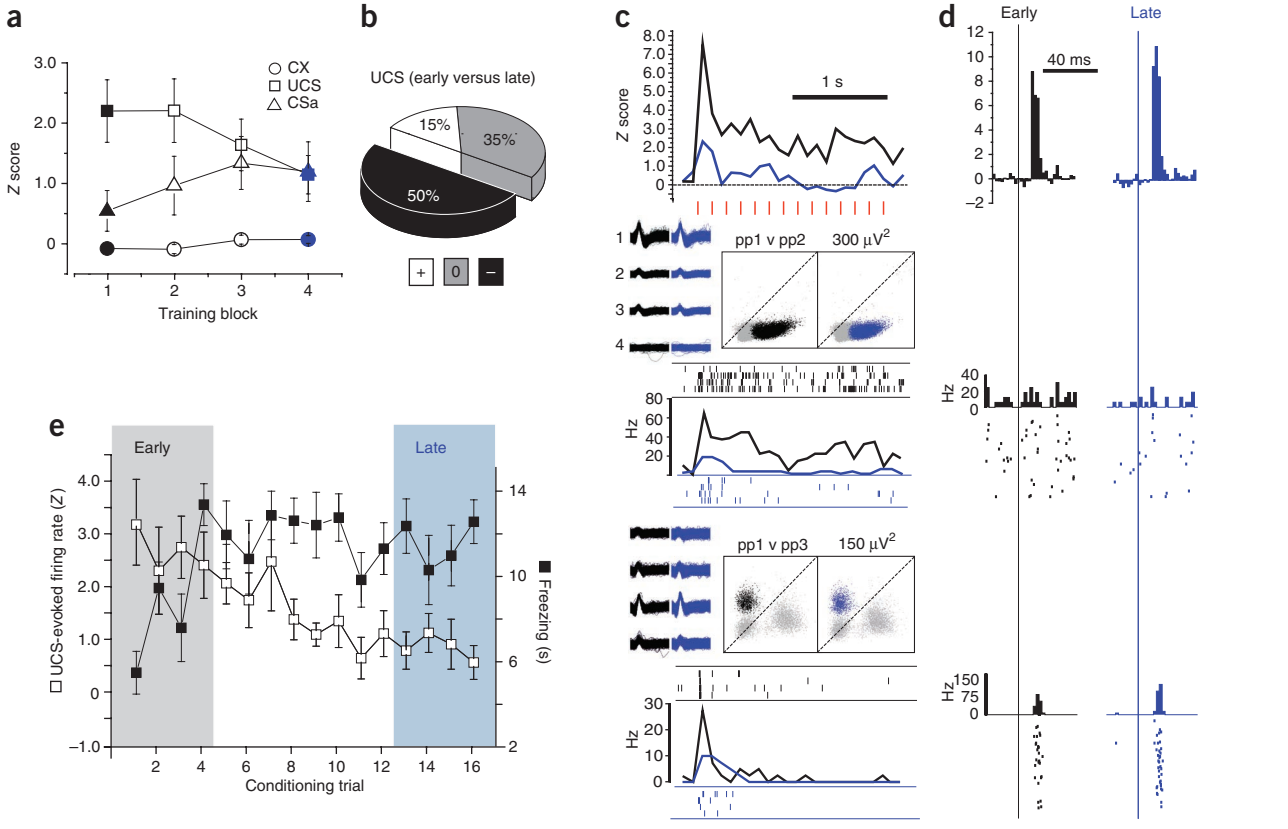

Figure 5 Activity of PAG neurons during acquisition of fear conditioning. (**a**) Normalized stimulus-evoked responses (*y* axis) averaged over the population of shock-responsive PAG neurons ($n = 20$) for each of the four conditioning trial blocks (four trials per block, 16 trials total). (**b**) Pie chart showing the percentage of shock-responsive PAG cells that significantly reduced (–), increased (+) or did not change (0) their UCS-evoked responses between the first (early) and last (late) conditioning trial block. (**c**) Top, PSTH (bin size = 100 ms) showing normalized activity during shock trains (individual shock pulses indicated by red hash marks) for early versus late conditioning trials, averaged over the subpopulation of PAG neurons that significantly reduced their shock-evoked response ($n = 10$). Bottom, PSTHs and spike rasters showing UCS-evoked responses for two example neurons from different rats along with waveforms and cluster plots as shown in **Figure 1e**. (**d**) Top, PSTHs (bin size = 2 ms) showing auditory responses (onset of white noise pip indicated by vertical line) during the early versus late conditioning trial block for the subpopulation of PAG cells shown in **c**. Bottom, PSTHs and spike rasters showing auditory responses for the same two example neurons shown in **c**. (**e**) Responses to the UCS (left *y* axis) on each of the 16 conditioning trials (averaged over the subpopulation of PAG cells that significantly reduced their UCS responsiveness during conditioning) are graphed alongside average freezing scores (right *y* axis) during the CSa period on each trial.

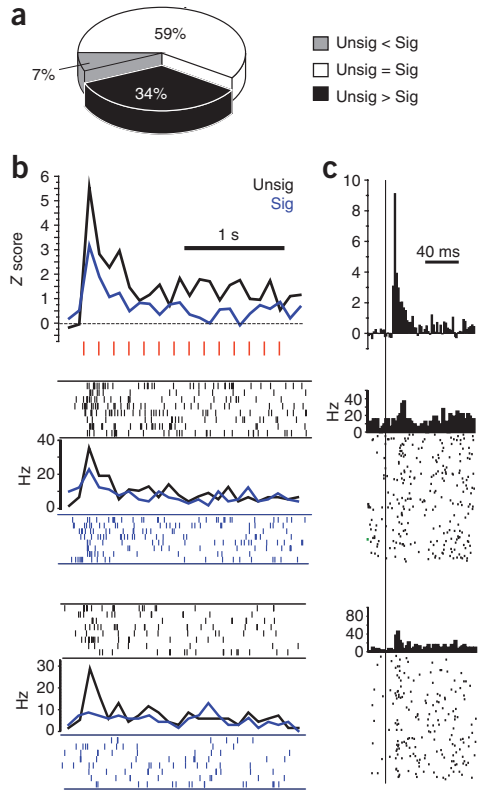

Figure 6 PAG neurons responded more to unsignaled than to signaled shocks. (**a**) Pie chart showing the percentage of shock-responsive PAG cells that responded significantly more to unsignaled than to signaled shocks (black), significantly more to signaled than to unsignaled shocks (gray) or the same to both types of shock (white). (**b**) Top, PSTHs (bin size = 100 ms) showing normalized activity during signaled and unsignaled shock trains (individual shock pulses indicated by red hash marks) for the subpopulation of PAG neurons ($n = 21$) that responded significantly more to unsignaled than to signaled shocks. Bottom, PSTH and spike rasters showing responses to signaled and unsignaled shocks for two example neurons from different rats. (**c**) Top, PSTH (bin size = 2 ms) shows normalized auditory responses (onset of white noise pip indicated by vertical line) during signaled trials for the subpopulation of PAG neurons shown in **b**. Bottom, PSTHs and spike rasters showing auditory responses for the two example neurons shown in **b**.

test, $P = 0.09$). As in the amygdala, the averaged response of these cells was inversely correlated with freezing to the CSa across conditioning trials ($r_{16} = -0.49$, $P = 0.05$), suggesting that attenuation of shock-evoked responses in PAG emerged as rats learned to expect the UCS (**Fig. 5e**).

After the initial conditioning session, responses to signaled and unsignaled shocks were recorded as described above for the amygdala recording experiments. A total of 93 PAG neurons were recorded from 13 rats, with at least one shock-responsive cell recorded from each rat. Significant shock-evoked responses were observed in 61 of 93 (66%) of these neurons and shock-evoked responses of these cells were larger to unsignaled than to signaled shocks ($t_{60} = 3.34$, $P = 0.001$). Analysis of individual neuron responses revealed that 34.5% (21 of 61) of the cells exhibited a larger response to the unsignaled UCS (**Fig. 6a–c**), whereas only 8% (4 of 61) of the cells exhibited a larger response to the signaled UCS; the remaining 54.5% (36 of 61) of the neurons responded similarly to signaled versus unsignaled delivery of the shock UCS (**Fig. 6a**). Thus, PAG neurons were about fourfold more likely to respond preferentially to the unsignaled UCS ($n = 21$) than to the signaled UCS ($n = 5$) and this was a significant bias for preferential responding to unsignaled shocks (two-tailed binomial test, $P = 0.0025$). Differential responses of PAG neurons to predicted versus unpredicted shocks were not attributable to differences in motor responses to the shocks (**Supplementary Fig. 12**). These results indicate that, similar to amygdala neurons, shock-evoked responding in PAG neurons is modulated by expectation.

DISCUSSION

Theory and evidence suggest that fear conditioning is instructed by a teaching signal that diminishes in intensity as expectation of the UCS increases[17,18,20,21,26,27,35,36]. Depolarization of amygdala neurons by an aversive UCS is thought to serve as the teaching signal that strengthens conditioned stimulus inputs onto amygdala neurons during fear learning[1,2,6] and our results suggest that UCS-evoked responses of neurons in both LAn and PAG are inhibited by expectation of the UCS during fear conditioning in rats.

We found that UCS-evoked responses in the LAn and PAG decreased over the course of training in a manner that was inversely correlated with increased freezing behavior (**Figs. 1** and **5**) and that this training regimen produced a reduction in the ability of a predicted UCS to support further fear conditioning (**Fig. 2d**). Following conditioning, amygdala and PAG neurons responded more robustly to shocks when they were presented unexpectedly than when they were signaled by the predictive CSa (**Figs. 2** and **6**). Finally, pharmacological inactivation of the PAG attenuated UCS-evoked responses in LAn neurons (**Fig. 3**) and impaired acquisition of fear conditioning (**Fig. 4**). These data indicate that amygdala and PAG neuronal responses to shock stimuli are negatively modulated by expectation and suggest that the PAG may relay expectancy-modulated shock information to amygdala neurons to instruct associative neural plasticity and support fear learning (**Supplementary Discussion**).

We found that some LAn neurons exhibited conditioned enhancement of their auditory-evoked responses and others did not, but both kinds of neurons were equally likely to exhibit inhibition of their UCS-evoked responses by expectation. This pattern of results is consistent with learning theories that postulate that a primary function of an expectation-modulated teaching signal is to regulate competition among associative learning elements (in this case, amygdala neurons)[17,37] (**Supplementary Discussion**). The opportunity to gain in associative strength may be lost once the UCS becomes expected, so only those amygdala neurons that strengthen their conditioned stimulus inputs early in conditioning (while the UCS is still unexpected) should ever be able to do so. A number of different factors may influence which subset of amygdala neurons succeed in strengthening their conditioned stimulus inputs, such as the availability of specific intracellular signaling molecules[30], or whether the UCS-responsive neurons receive convergent inputs from sensory neurons that encode the conditioned stimulus (LAn neurons receive inputs from multiple sensory modalities and many cells respond exclusively to one modality[38]). To serve as a general-purpose teaching signal for fear learning over a wide range of conditioned stimulus modalities and learning contexts, the expectancy-modulated UCS signal should be broadly distributed throughout the network of amygdala neurons, so that it can instruct plasticity in whichever subset of neurons are best suited for storing associative memories in a given learning situation. Thus, enhancement of conditioned stimulus–evoked responses should be observed only in a subset of a larger population of neurons that exhibit inhibition of UCS processing by expectation, as we observed in our experiments.

Much evidence has suggested that PAG is an output structure for various fear-conditioned responses, including freezing, analgesia and vocalization, as well as unconditioned reactions to aversive unconditioned stimuli, such as shock[31,32,34,39]. Consistent with these results, we found that PAG inactivation reduced the expression of both conditioned fear responses and unconditioned reflex responses to the shock UCS (**Supplementary Figs. 6** and **9**). However, if PAG serves only as an output pathway for fear conditioning, then PAG inactivation should impair expression, but not acquisition, of fear conditioning. Contradicting this, we found that fear acquisition was impaired by pre-training inactivation of PAG (**Fig. 4**). Pre-training PAG inactivation may have reduced fear learning by blocking local plasticity in the PAG, a possibility that is suggested by prior evidence[40], but this would not explain why this manipulation reduced shock-evoked responding in amygdala neurons. A parsimonious interpretation of the data is that the PAG may serve multiple functions during fear conditioning, mediating both the expression of fear responses and the transmission of teaching signals to the amygdala to regulate amygdala plasticity and the resultant acquisition of fear learning.

The PAG is anatomically well-positioned to receive afferent sensory information about the shock UCS, as it receives major input from nociceptive and somatosensory neurons in the medullary and spinal dorsal horn[41,42]. Moreover, stimulation of PAG neurons can substitute for a noxious UCS to support fear conditioning[43], suggesting that output from PAG is sufficient to generate an aversive teaching signal that instructs associative plasticity in the amygdala. Direct projections from PAG to LAn are sparse[44], but there are a number of nociceptive and neuromodulatory brain regions that receive PAG afferents and project to the LAn, such as the intralaminar thalamic nuclei, anterior cingulate cortex, hypothalamus, locus coeruleus and the ventral tegmental area[45–49]. Some of these PAG targets are involved in the acquisition of fear conditioning[12–16,50], so PAG may participate in relaying UCS signals to the amygdala indirectly via one or more of these brain regions. A more detailed understanding of these aversive teaching signal pathways will be important for guiding future investigations of the neural circuitry mediating fear conditioning, as well as other forms of learning that are instructed by aversive events.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

All authors contributed to the planning and design of the study. Data collection was performed by J.P.J. and J.W.T. Data analysis and writing of the manuscript were performed by J.P.J., J.W.T. and H.T.B. The neurophysiology and fear conditioning experiments were conducted in the laboratory of H.T.B. and the blocking experiments were conducted in the laboratory of J.E.L.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

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 isoflourane anesthesia, rats were implanted with a pair of insulated stainless steel wires (75 μm in diameter) beneath the skin of each eyelid for delivering the periorbital shock unconditioned stimulus. In addition, rats were implanted with recording electrodes, intracranial infusion cannulae, or both (see below). Electrodes and cannulae were fixed in place by securing screws and bone cement and rats were given at least 5 d to recover after surgery before experiments began.

Single-unit recording electrodes. The left or right hemisphere (counterbalanced) of either the LAn or PAG was implanted with a microdrive containing six tetrode bundles made from 0.0007-inch formvar-insulated nichrome wire (Kanthal). For LAn recordings, electrode tips were placed just above the lateral tip of LAn at 3.0 mm posterior, 5.3 mm lateral and 7.0 mm ventral to bregma. For PAG recordings, tips were placed just above the dorsal column of PAG at 7.65 mm posterior, 0.75 mm lateral and 3.5 mm ventral to bregma.

Intracranial infusion cannula. Rats in PAG inactivation experiments were bilaterally implanted with a pair of stainless steel 26G guide cannulae filled with 33G dummies projecting 0.5 mm from the guide tips. Guide tips were placed at 7.8 mm posterior, ± 0.75 mm lateral and 4.3 mm ventral to bregma, so that injectors protruding 1.5 mm from the guide would later deliver infusions into the ventrolateral PAG at 5.8 mm ventral to bregma.

Fear conditioning. Throughout experimental sessions (except for the blocking experiment), rats foraged freely for 20-mg purified food pellets (Bioserv) dropped randomly from an overhead dispenser. Three different contexts were used in the experiment: a circular platform made of black plastic cleaned with lemon-scented solution and surrounded by three white walls and a white curtain (70 cm diameter), a square platform (70 × 70 cm) made of gray-painted wood cleaned with mint-scented solution and surrounded by three black walls and a black curtain, and a square platform (70 × 70 cm) made of gray-painted wood cleaned with Windex and surrounded by three gray walls and an open side with no curtain. For all electrophysiology experiments, rats were run on the third platform. For PAG behavioral experiments (**Fig. 4**), rats were randomly assigned to a platform at the start of the experiment, where they learned to pellet chase over 5 d of pre-exposure. Rats in PAG behavioral experiments began fear conditioning on the first day after pre-exposure, whereas rats in neural recording experiments began fear conditioning on the first day that well-isolated neurons were encountered in the target area. In all experiments, the conditioned stimulus 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 unconditioned stimulus was a train of 2.0-mA shock pulses, each lasting 2.0 ms, delivered to the eyelid contralateral from the recording hemisphere at a rate of 6.66 Hz for 2 s. During paired training trials, the first shock pulse was delivered 300 ms after the offset of the final (twentieth) CSa pip. The inter-trial interval was uniformly random between 180 and 240 s.

For PAG behavioral experiments, rats were placed on their assigned platform and presented with six CSa presentations on the first day after pre-exposure to measure baseline freezing responses to the context and CSa. Rats received either MUS or vehicle (0.4 μl, 0.25 mg ml^{-1} over a period of 100 s) microinjections into the PAG or the lateral off-site control site 24 h later. The rats received 16 pairings on the previously assigned platform 20 min after microinjection and were then returned to their home cages. The rats were placed on a novel platform 6 d later (to allow drug effects to wear off) for the first time and presented with six test presentations of the CSa alone to assess conditioned freezing responses.

For electrophysiology studies, fear conditioning was as described above, except that the pre-training test session, fear conditioning session and post-training test session all occurred on the same day. The pre-training test session was followed immediately by the fear conditioning session, after which rats rested in their home cages for 1 h before returning to the platform for the post-training test session. On subsequent days, electrodes were advanced until new cells were isolated, at which time rats were given a 'signaled-unsignaled' session in which eight unsignaled shocks (not preceded by the CSa) and eight signaled shocks (preceded by the CSa) were presented randomly. Between the original conditioning session and all of the 'signaled-unsignaled' sessions, rats were retrained with the standard fear conditioning session (16 pairings). Electrode advancement and recording sessions continued in this manner until the electrodes were no longer in the amygdala.

For the blocking experiment, rats were conditioned in a sound-isolating chamber and did not chase food pellets. On day 1, rats were given six presentations of a flashing light CSv (at the same frequency and duration as the CSa pips described above) alone followed by six presentations of the CSa alone. On day 2, the blocking group received 16 pairings of the CSa and eyelid shock UCS (identical to prior experiments), whereas the naive group received no treatment. On day 3, all rats received 16 pairings of a compound conditioned stimulus consisting of the CSa and CSv (at the same frequency and duration as the CSa) and the eyelid shock unconditioned stimulus. The rats were given six presentations of the CSv alone 24 h later.

Behavior tracking. Freezing and movement (except in the blocking experiment) were measured by an overhead video tracking system that has been described elsewhere[28]. Briefly, the video tracker sampled (at 30 Hz) the position of two colored light-emitting diodes attached to the rat's headstage throughout the experiments and movement data was extracted by taking the time derivative of the sampled position data. Custom software written in MATLAB (Mathworks) was used to analyze the movement data and extract freezing and head-jerking responses. Freezing on each trial was computed as the summed duration of episodes lasting at least 300 ms during which the rat's movement speed was less than 10 cm s^{-1}. Our fear conditioning protocol yielded lower freezing scores than other protocols for a variety of reasons, including the fact that the shock was localized to a small somatic area and that there were competing behavioral responses (that is, pellet chasing). Unconditioned head-jerking responses were quantified by averaging the rat's head velocity (in cm s^{-1}) during the shock train.

For the blocking experiment, the rat's behavior was recorded on video and a rater who was blind with respect to the treatment group scored rats' behavioral freezing during the pre–conditioned stimulus period (20 s preceding conditioned stimulus onset) and during the conditioned stimulus period offline using a digital stopwatch. Freezing was defined as the cessation of all bodily movement with the exception of respiration related movement.

Single-unit recording. After rats had recovered from electrode implantation surgery, daily screening sessions were conducted in which electrode tips were slowly advanced (<200 μm per day) into the targeted brain area (LAn and basal nucleus complex or PAG). Neurons were tested for contralateral eyelid–evoked shock responsiveness using mild single-shock pulses. If no shock responsive neurons were encountered, the electrodes were advanced.

Cluster analysis. Single-unit recordings were obtained using a 32-channel data acquisition system (Neuralynx). Offline cluster cutting was performed manually using Neuralynx SpikeSort 3D software. To be included in the study, spike trains had to exhibit a refractory period of at least 1 ms and a mean spike amplitude of at least 70 μV. Spike waveforms and cluster boundaries were inspected to ensure that they remained stable throughout the recording session (which lasted between 0.75–2.5 h) for cells included in the data analysis.

Recording and inactivation experiments. To assess the effects of PAG inactivation on amygdala responses, bilateral MUS infusions were delivered into PAG (0.4 μl, 0.25 mg ml^{-1} over a period of 100 s) immediately after a drug-free signaled-unsignaled shock session. The rat was placed back in its home cage for 20 min before returning to the platform for a second signaled-unsignaled shock session to examine the effects of PAG inactivation on amygdala activity.

Data analysis. The value of each PSTH bin was computed $S_i = C_i / N$, where N is the number of trigger events (trials or stimuli) for the PSTH and C_i is the cumulative number of spikes in the i^{th} bin across trigger events. Prior to population averaging, the PSTH of each cell was normalized by converting S_i values to z scores using the formula $Z_i = \frac{(S_i - \mu)}{\sigma}$, where μ and σ are the mean and s.d., respectively, of all S_i values in a set of baseline bins. PSTHs that were triggered once per trial had a bin width of 100 ms and the baseline bins for normalization

were the 200 S_i values from the 20 s pre-conditioned stimulus period on signaled shock trials, or the 20-s period before when the omitted conditioned stimulus would have occurred for unsignaled shock trials. PSTHs that were triggered once per pip stimulus had a bin width of 2 ms, and the baseline bins for normalization were the 250 S_i values from the 0.5-s period preceding the onset of each pip. Baseline PSTHs were computed from all trials in the session for normalization of PSTHs that included only subsets of trials in a session (such as early conditioning trials, unsignaled shock trials, etc.).

Shock responsiveness of individual neurons. A neuron was considered to be responsive to shocks during a block of trials if the 20 Z_i values from bins in the 2-s shock train period of its normalized PSTH met one of three criteria: at least one bin with $Z_i > 3$, two or more consecutive bins with $Z_i > 2$, or three or more consecutive bins with $Z_i > 1$. For conditioning sessions, a cell was considered to be shock responsive if one of the criteria was met by the normalized PSTH for any trial block in the session ($N = 4$ trials per block). For signaled-unsignaled sessions, a cell was considered to be shock responsive if one of the criteria was met by the normalized PSTH of either signaled ($N = 8$) or unsignaled ($N = 8$) shock trials. All bins meeting one of the three response criteria in a neuron's PSTH from any trial block in a conditioning session, or either trial type in a signaled-unsignaled session, were combined to define a region of interest (ROI) window for inferential comparisons of the neuron's responses to stimuli under differing conditions.

Inferential statistics for individual neurons. To determine whether an individual neuron's shock responsiveness differed between two conditions (early versus late conditioning trials or signaled versus unsignaled shock trials), we counted spikes in a 100-ms window following each shock pulse whose onset occurred in the neurons' ROI window, and an independent *t* test compared spike counts from the two conditions (so the degrees of freedom for the *t* test were $df = 2(T \times P) - 2$, where T is the number of trials per condition and P is the number of pulses per trial occurring in the ROI). The neuron was considered to respond differently to the stimulus in each condition if $P < 0.05$ (two tailed). To compare pip responsiveness of amygdala neurons before and after conditioning, we used the 10–30 ms time window following the onset of each white noise pip as the ROI.

Inferential statistics for neural populations. To determine whether a population of neurons changed their responsiveness to a stimulus between two conditions, we averaged the Z_i values of each neuron in the population in a specified time window of the normalized PSTH to derive the response in each of the two conditions and a paired *t* test or repeated-measures ANOVA was performed (with N = number of cells) to compare the responses during different conditions.

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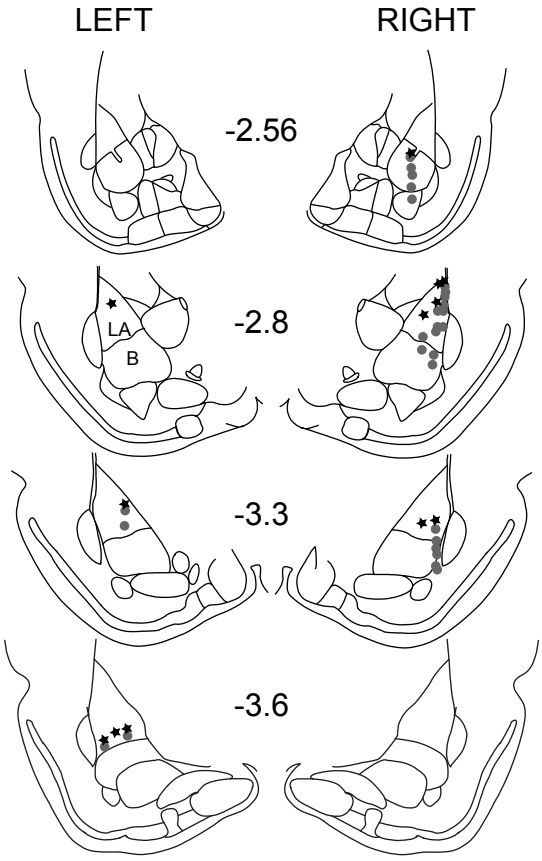
Neural substrates for expectation-modulated fear learning in the amygdala and periaqueductal gray

Johansen, J.P., Tarpley, J.W., LeDoux, J.E. & Blair, H.T.



Supplementary Figure 1: Histological reconstruction of recording sites in LA/B for cells recorded during conditioning (black stars) and later sessions in which signaled and unsignaled US's were presented randomly (gray circles). Coronal sectionsshow histological reconstructions of recording sites in LA/B (adapted from (Paxinos and Watson, 1982)).

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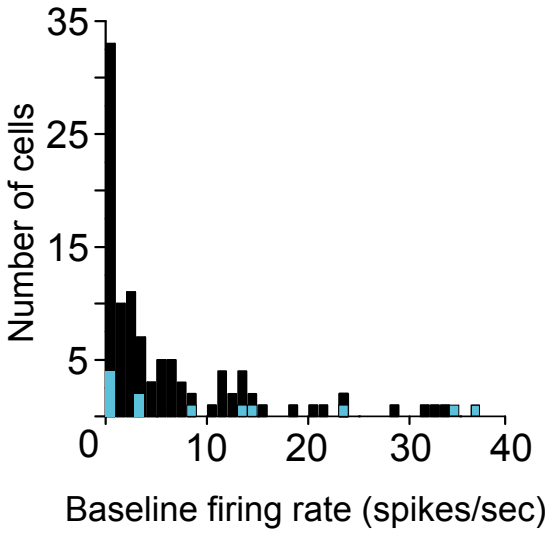

Supplementary Figure 2: Frequency distribution of baseline firing rates for all cells recorded in the LA/B (n=104; bin size = 1 Hz). The LA and B nuclei contain glutamatergic principle cells as well as GABA-ergic interneurons, with interneurons typically exhibiting higher firing rates than principle cells. The baseline firing rates of cells that diminished their US responsiveness during conditioning (light blue) were broadly distributed across the full range of observed firing rates, providing evidence that diminution of US-evoked responses was similarly prevalent in both principal cells and interneurons.

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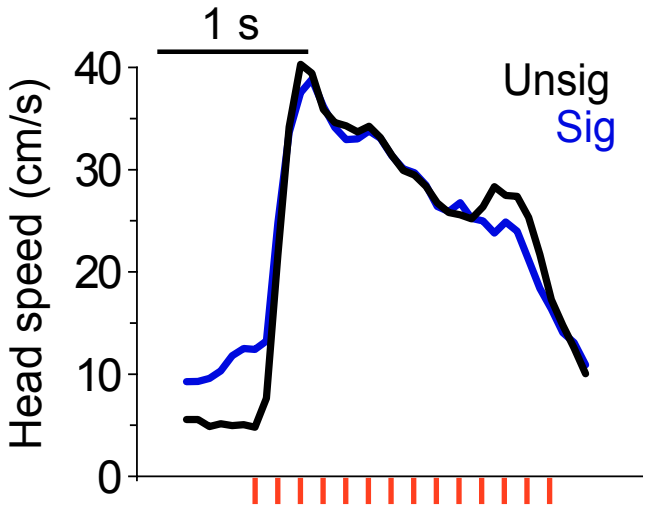

Supplementary Figure 3: Unconditioned responses to signaled (blue) versus unsignalled (black) shocks during LA/B recordings. Graphs show mean movement speed during the train of shock pulses (red hash marks) averaged over recording sessions (n=13, 8 rats) during which LA/B neurons that responded preferentially to unsignaled shocks (n=15) were recorded. Head responses to signaled vs. unsignaled shocks were compared by averaging the head movement speed across the entire 2.0 s of the shock train for each session, and performing a paired t-test to compare the averaged movement speeds during signaled vs. unsignaled shocks. There was no difference in the magnitude of movement responses to signaled vs. unsignaled shocks (paired t_{12}=.45, p=.65) during recordings of LA/B neurons that responded preferentially to unsignaled shocks.

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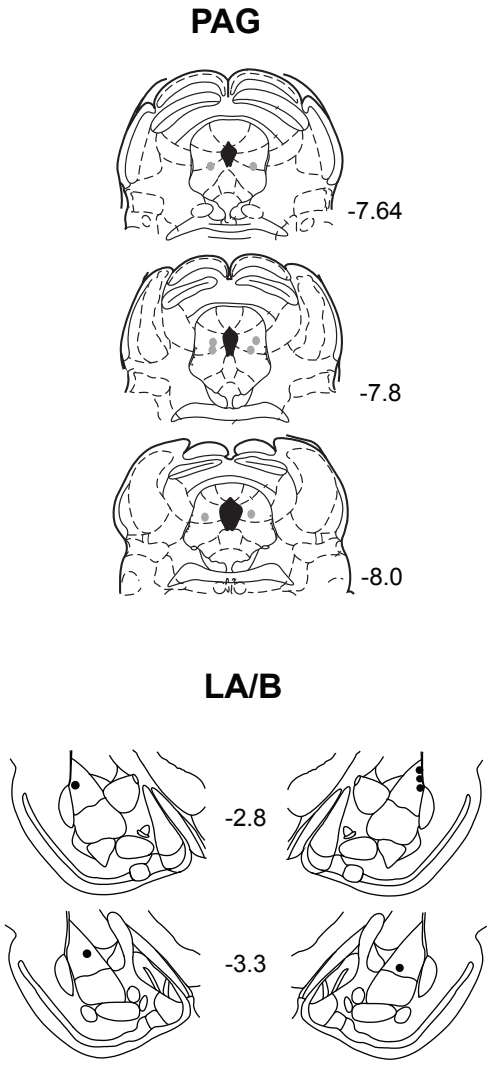

Supplementary Figure 4: Histological reconstruction of recording sites in LA/B and injection sites in PAG for experiments in which LA/B cells were recorded (black circles) during presentation of signaled and unsignaled US's before and after microinjection of muscimol into the PAG (microinjection sites denoted as grey circles). Coronal sections show histological reconstructions of recording sites in LA/B and injections sites in the PAG.

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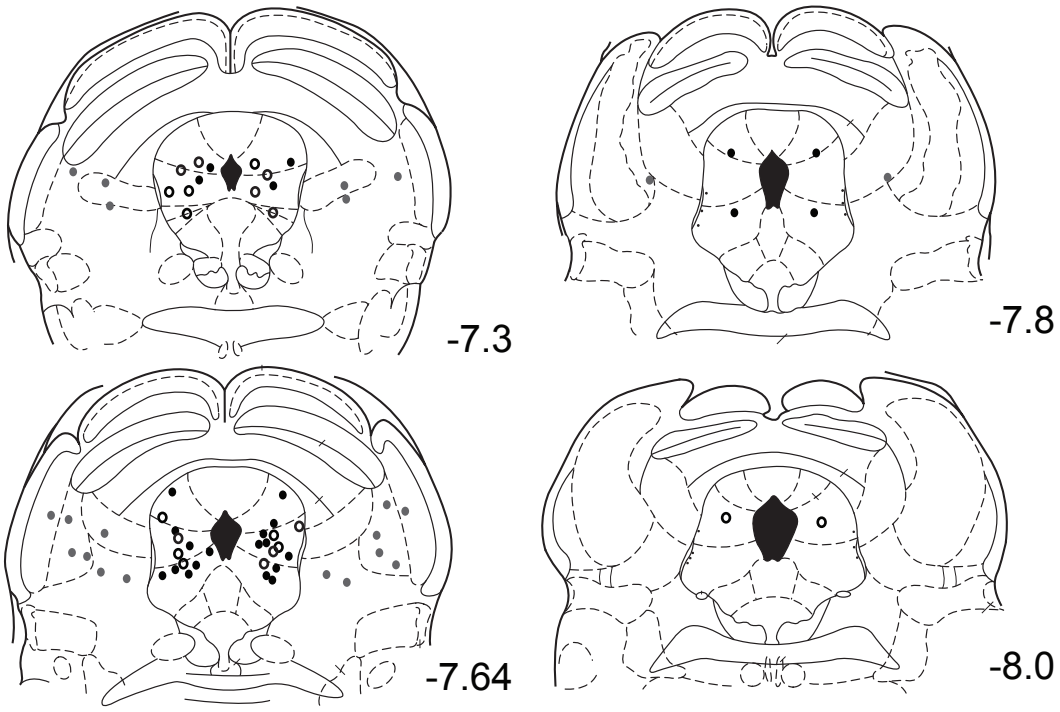

Supplementary Figure 5: Histological reconstruction of PAG injection sites for behavioral experiments. Coronal sections show histological reconstructions of injection sites in PAG (adapted from(Paxinos and Watson, 1982) represented by closed (MUS) and open (VEH) circles and lateral to the PAG (Offsite, gray circles).

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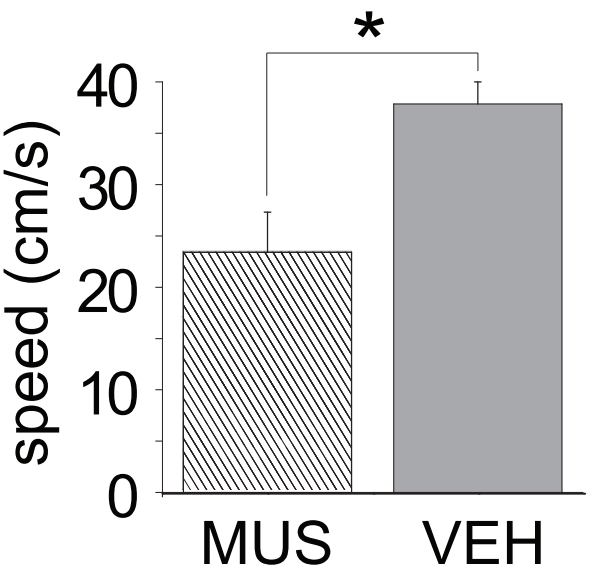
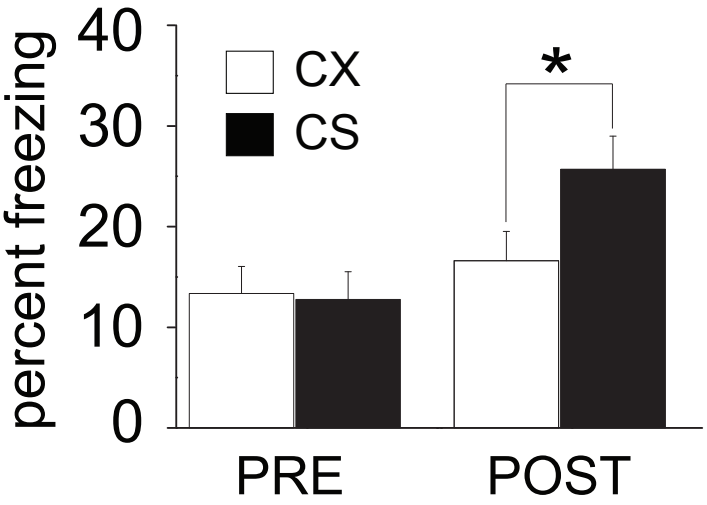
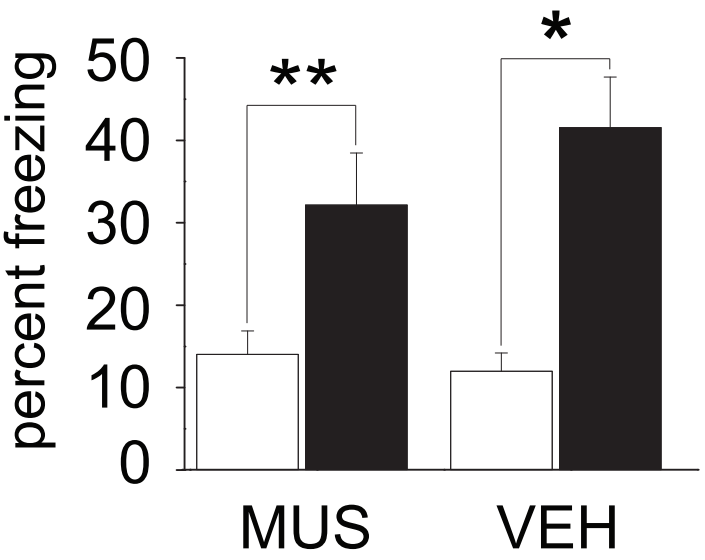


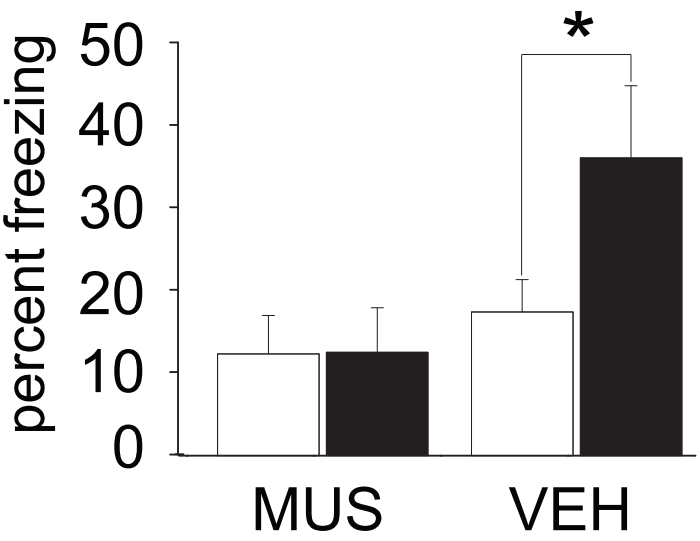
Supplementary Figure 6: Effects of PAG inactivation on unconditioned responding to the US during acquisition. Movement speed (y-axis) during the shock train after infusions of MUS versus VEH into PAG. MUS treated animals exhibited lower URs compared with VEH treated animals (t_{21}= 3.15, p=0.005)



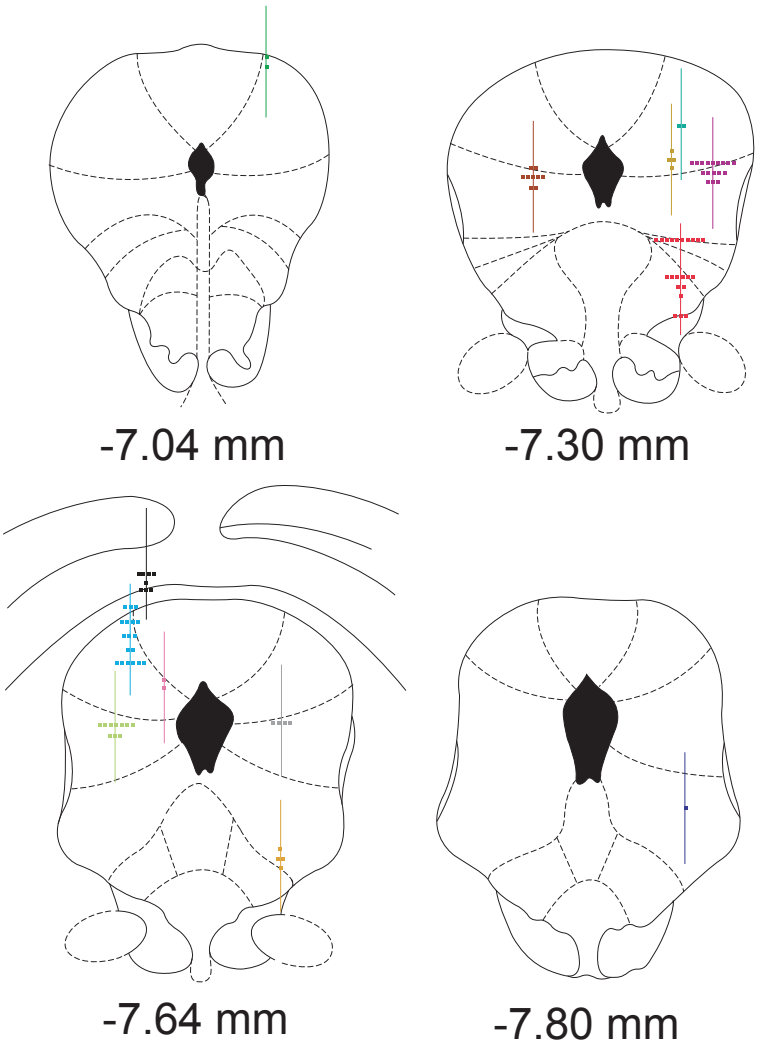
Supplementary figure 7: effect of off-site PAG inactivation on the acquisition of fear conditioning Freezine measured 6 days after conditioning in the off-site control group which had received pre-training infusions of MUS into sites lateral to the PAG. A 2X2 repeated measures ANOVA revealed a main effect ($F_{1,20}=4.417$, $p=0.049$) of session (pre vs. post conditioing) and a significant interaction ($F_{1,20}=9.953$, $p=0.005$) between session and stimulus (CX vs. CS). Unplanned post-hoc comparisons showed that rats froze more the CS than to the CX during the drug free post conditioning test ($p=0.0005$), *. See Suppl. Fig. 5 for injection site reconstruction.



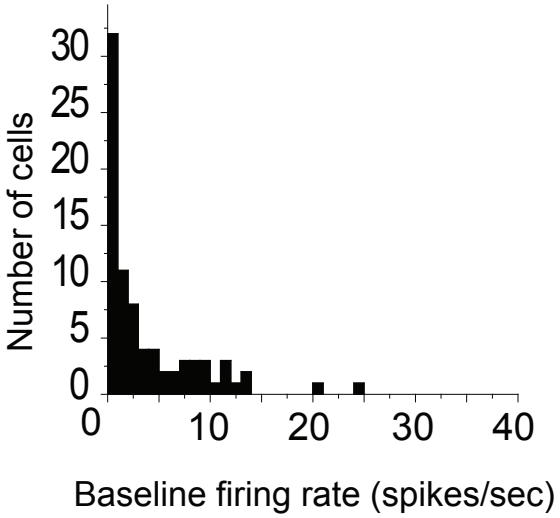
Supplementary Figure 8: Effects of prior PAG inactivation on re-training of fear conditioning Some rats from each group which had been previously fear conditioned (MUS and VEH, see Fig. 4) were given a drug-free retraining session (16 CS-US pairings) immediately after the test trials on the novel platform, then 24 h later they were placed on another novel platform (the one remaining platform not yet visited) for a re-test session of 6 CS alone presentations . Freezing measured 24 hours after drug-free retraining of rats that had previously received intra-PAG infusions of MUS or VEH. A 2X2 ANOVA demonstrated a main effect ($F_{1,21}=28.532$, $p=2.7\text{-}5$) of stimulus condition (CX vs. CS), but no effect ($F_{1,21}=0.519$, $p=0.479$) of group (MUS vs. VEH) and no interaction ($F_{1,21}=1.637$, $p=0.215$) between group and stimulus. Unplanned post-hoc comparisons showed that VEH ($p=0.0008$, *) and MUS ($p=0.009$, **) rats both froze significantly more to the CS than to the CX following drug-free retraining.



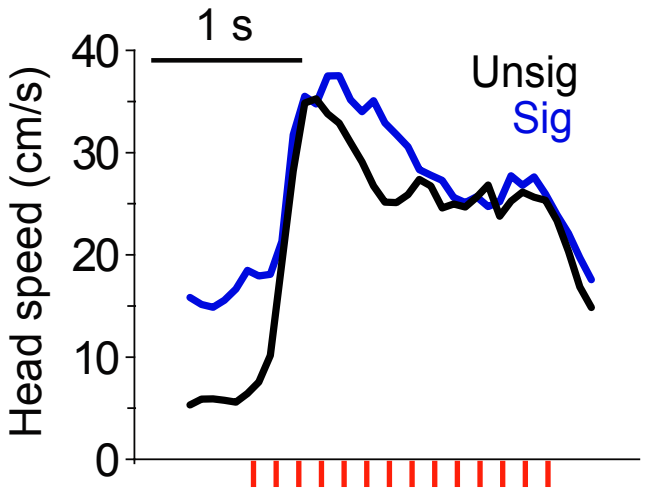
Supplementary Figure 9: Effects of PAG inactivation on expression of previously learned fear conditioning. Some of the rats from the PAG MUS experiments were retrained drug free and infused with either MUS or VEH (as described above) and given another test session. Freezing in well-trained rats during a test session conducted immediately after intra-PAG infusions of MUS or VEH. A 2X2 repeated measures ANOVA revealed a significant interaction ($F_{1,12}=5.676$, $p=0.034$) between infusion (MUS vs. VEH) and stimulus condition (CX vs. CS). Unplanned post-hoc comparisons demonstrated that while VEH rats froze significantly more to the CS than the CX ($p=0.005$, *), there was no difference in CS compared with CX freezing in MUS rats ($p=0.972$).



Supplementary Figure 10: Histological reconstruction of recording sites in PAG. Colored vertical lines demonstrating electrode tracks from each of 13 rats where each color represents and individual rat. The vertical lines represent the plane of the electrode track, the squares represent the # of cells recorded at each depth along a track, and the point at which the squares intersect the line represents the recording site. All cells were recorded in the hemisphere contralateral from the shocked eyelid.



Supplementary Figure 11: Frequency distribution of baseline firing rates for all cells recorded in the PAG (n=114; bin size = 1 Hz)



Supplementary Figure 12: Unconditioned responses to signalled (blue) versus unsignalled (black) shocks during PAG recordings. Graphs show mean movement speed during the train of shock pulses (red hash marks) averaged over recording sessions (n=15, 7rats) during which PAG neurons that preferentially to unsignaled shocks (n=21) were recorded. Head responses to signaled vs. un-signaled shocks were compared by averaging the head movement speed across the entire 2.0 s of the shock train for each session, and performing a paired t-test to compare the averaged movement speeds during signaled vs. unsignaled shocks. There was no difference in the magnitude of movement responses to signaled vs. unsignaled shocks (paired t_{14}=1.3, p=.21) during recordings of PAG neurons that responded preferentially to unsignaled shocks.

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Supplementary Discussion

Modulation of aversive stimulus processing by expectancy

Theory and evidence have suggested that presentation of a well-trained CS activates outputs from the amygdala to PAG, which in turn triggers descending analgesia via the brainstem that inhibits aversive sensory processing in the dorsal horn of the spinal cord and brainstem trigeminal system during anticipation of the US [1-8]. If it is true that expectation inhibits US processing via analgesia at the level of the spinal and trigeminal systems, then this inhibition should be observable at all subsequent levels of the neuraxis including the PAG and LA, in agreement with our present findings. However, if descending inhibition of the spinal-trigeminal system requires the PAG, then PAG inactivation might be expected to disinhibit US processing and thereby enhance the acquisition of fear conditioning. Contradicting this, we found that PAG inactivation reduced shock-evoked responding in LA neurons and impaired fear conditioning. As noted above, one possible explanation for this could be that PAG is not only a source of descending inhibition onto spinal/trigeminal circuits, but also an important relay center for ascending projections that transmit aversive teaching signals to the amygdala.

Although prior theories have proposed that inhibition of US processing by expectation may occur at the level of the spinal/trigeminal system [1,2,9], such inhibition might also (or instead) occur at higher levels of sensory processing[9]. Supporting this possibility, we found no difference in animals' unconditioned reflex responses to predicted versus unpredicted shocks (Supplementary Fig. 3), suggesting that shock signals were processed normally (and not inhibited by expectation) in the motor reflex

arc of the trigeminal system. Moreover, it has been shown that a well-trained CS which predicts shock to one eyelid can block fear conditioning, but not eyeblink conditioning, when a novel CS is presented in compound with the well trained CS and both are paired with shock to the opposite eyelid [10]. This implies that US processing was intact at the level of the trigeminal dorsal horn during the compound training phase (because eyeblink conditioning was not blocked), and that US -evoked teaching signals that instructed the fear system were inhibited by expectation independently from signals that instructed the eyeblink conditioning circuit. If so, then inhibition of the US-evoked teaching signal for fear conditioning may occur in brain regions downstream from the spinal/trigeminal system, possibly within the PAG itself. An important question for future research will be to identify the anatomical loci where US processing is inhibited by expectation in the fear circuit.

Expectancy modulation of US processing and computational models

The modulation of aversive US processing in LA neurons during fear conditioning is unlikely to be due to a non-associative mechanism (such as cross-modal sensory adaptation) whereby presentation of an auditory stimulus (the CS) diverts animals' attention away from the US, as early in conditioning when the CS was presented before the US (which should divert attention away from the US according to this hypothesis) the shock elicited a robust response in LA neurons. In addition, it is not likely that the observed reduction in shock-evoked responding in LA neurons during conditioning results from non-associative habituation of US-evoked responding, as LA neurons responded more to predicted than unpredicted shocks in well trained animals.

Thus it is likely that expectancy modulates US processing during fear conditioning as proposed by computational models[11,12]. These computational theories posit that both increases and decreases in associative strength are instructed by an expectation modulated 'prediction error' signal that measures the difference between actual and expected reinforcement in a bidirectional manner. The shock US-evoked response in LA neurons reported in the present study appears to encode some aspects of the prediction error signal proposed by these models. However, according to these theories, a prediction error signal should have a positive sign when an unexpected reinforcer is presented (consistent with the differential LA and B neuronal response to predicted and unpredicted shocks reported in the present study) and a negative sign when an expected reinforcer is omitted [11,12]. Midbrain dopamine neurons appear to encode this type of multidirectional prediction error signal[13,14](though DA neurons clearly do not represent negative and positive prediction errors symmetrically through firing rate). In the present study, however, although it was clear that US-evoked increases in firing rate in LA neurons was attenuated when the shock was predicted (thus coding a positive prediction error), there was not a significant inhibition of firing rate in these neurons by omission of expected shocks (thus they did not encode a negative prediction error). Supporting this, a recent study found limited inhibition of amygdala neurons when an expected aversive US was omitted during an eyeblink conditioning task in primates[15]. Thus, although somatosensory processing in LA neurons does appear to be modulated by expectation, LA neurons do not encode all aspects of a prediction error as set forth in the computational models. Based on what we know about fear conditioning, however, this finding may not be surprising. Positive prediction errors are thought to instruct initial

learning and negative prediction errors are thought to instruct inhibitory learning including the reversal of associations formed during the initial learning experience (i.e. extinction)[11,12,16]. While the prediction error term in these computational models provides a simple mathematical mechanism for associative fear learning, the neural implementation of this process may be more complicated and the positive and negative components of this equation may be mediated by at least partially separate neural circuits[17-20]. This is in fact plausible in the fear conditioning system as the neural plasticity mediating the acquisition of the fear learning is thought to occur in separate neural populations (pyramidal cells of the LA) from those in which the plasticity mediating the extinction or reversal of fear learning occurs (the medial prefrontal cortex and amygdala interneurons) [21-23]. Thus in the fear conditioning circuit, prediction error instructive signalling may be mediated by two partially separable systems; one which instructs plasticity of CS inputs onto LA neurons for the formation of fear memories and another which instructs plasticity in circuits mediating extinction learning.

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