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

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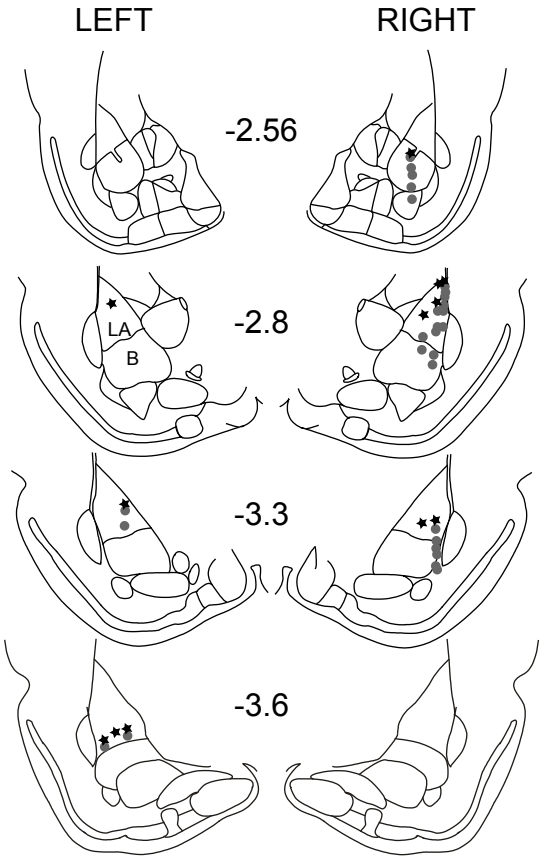
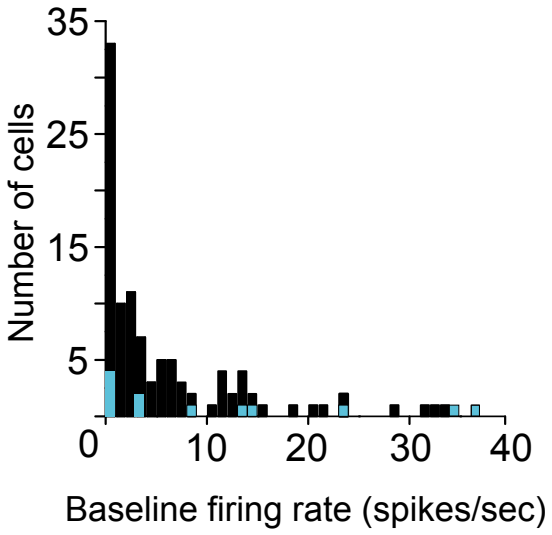
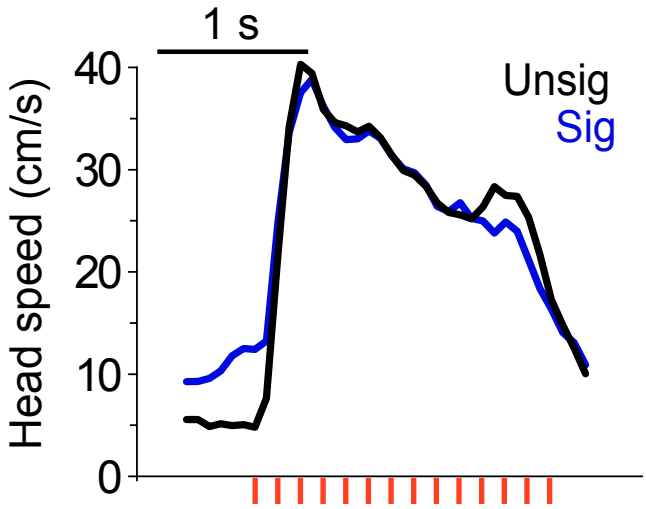


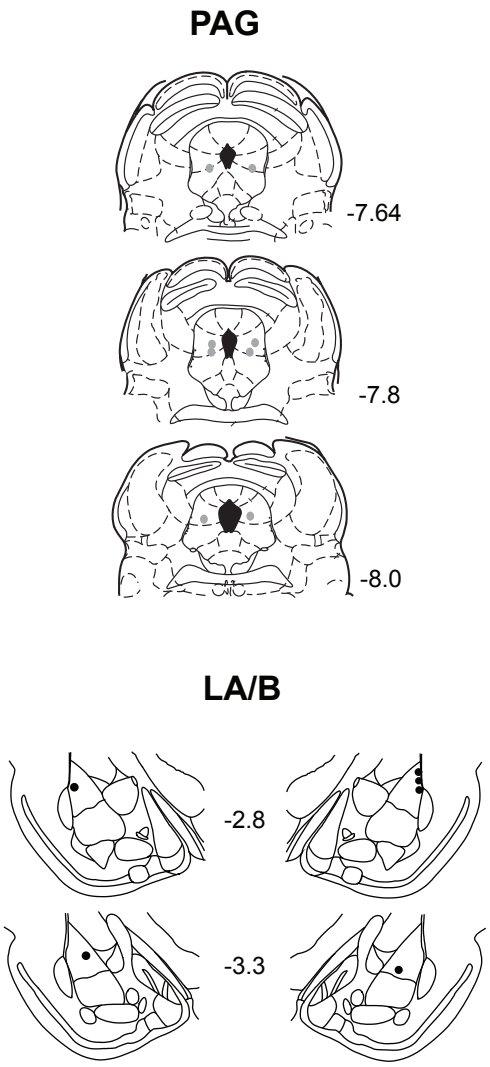
Supplementary Figure 1: Histological reconstruction of recording sites in LA/B for cells recorded during conditioning (black stars) and later sessions in which signaled and unsignaled US's were presented randomly (gray circles). Coronal sectionsshow histological reconstructions of recording sites in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



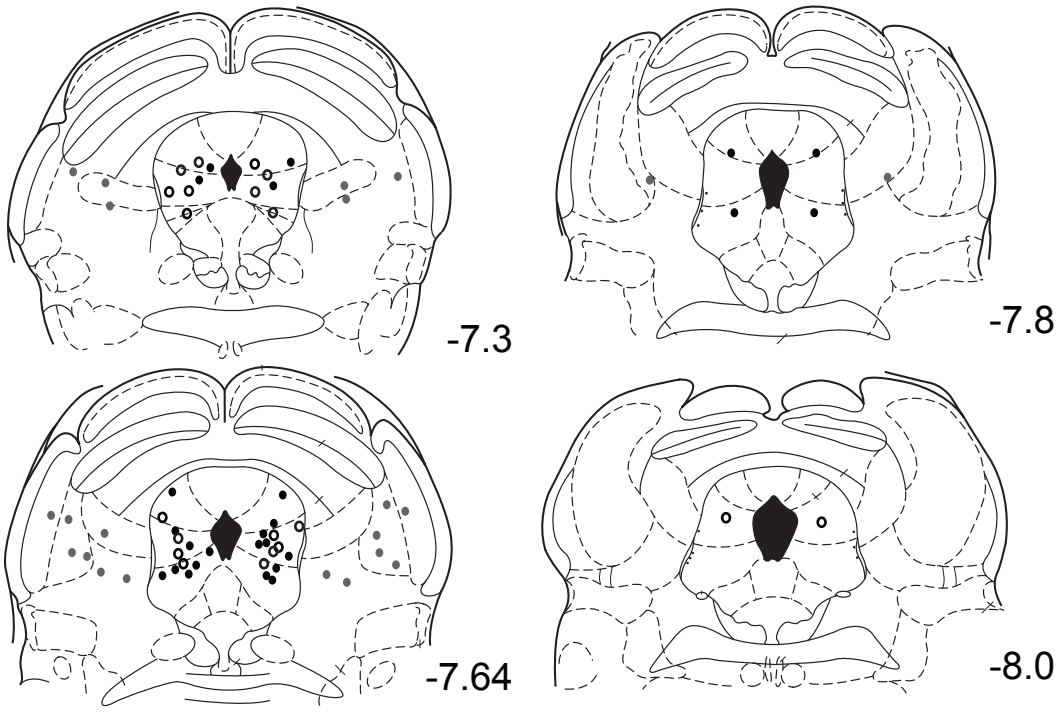
Supplementary Figure 2: Frequency distribution of baseline firing rates for all cells recorded in the LA/B (n=104; bin size = 1 Hz). The LA and B nuclei contain glutamatergic principle cells as well as GABA-ergic interneurons, with interneurons typically exhibiting higher firing rates than principle 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.



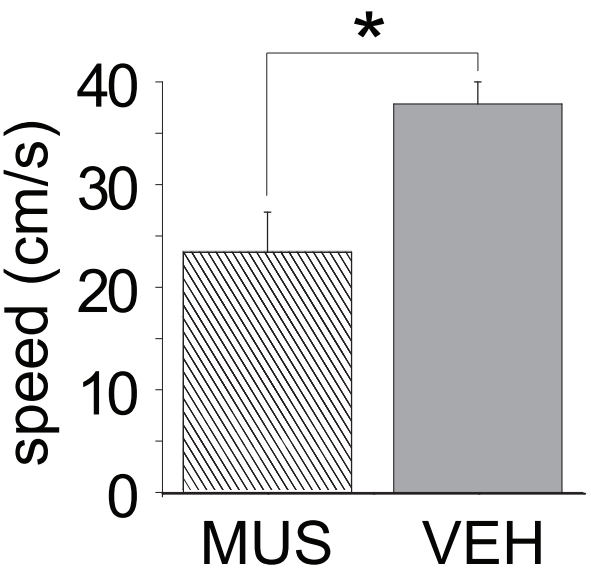
Supplementary Figure 3: Unconditioned responses to signaled (blue) versus unsignalled (black) shocks during LA/B recordings. Graphs show mean movement speed during the train of shock pulses (red hash marks) averaged over recording sessions (n=13, 8 rats) during which LA/B neurons that responded preferentially 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.



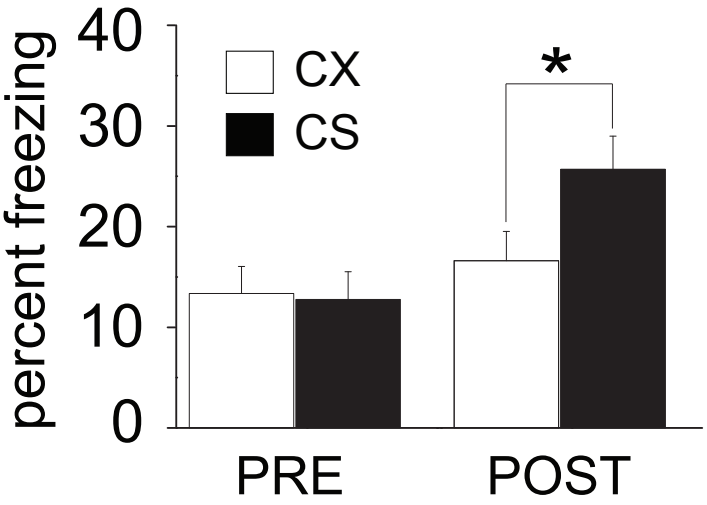
Supplementary Figure 4: Histological reconstruction of recording sites in LA/B and injection sites in PAG for experiments in which LA/B cells were recorded (black circles) during presentation of signaled and unsignaled US's before and after microinjection of muscimol into the PAG (microinjection sites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



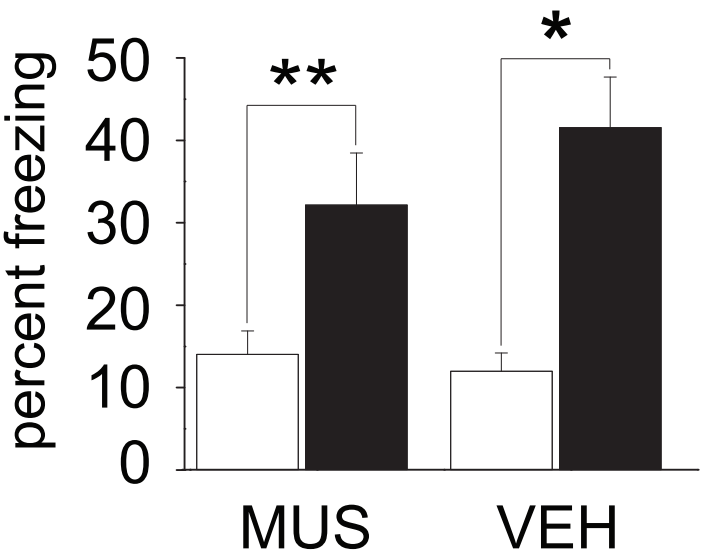
Supplementary Figure 5: Histological reconstruction of PAG injection sites for behavioral experiments. Coronal sections show histological reconstructions of injection sites in PAG (adapted from(Paxinos and Watson, 1982) represented by closed (MUS) and open (VEH) circles and lateral to the PAG (Offsite,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



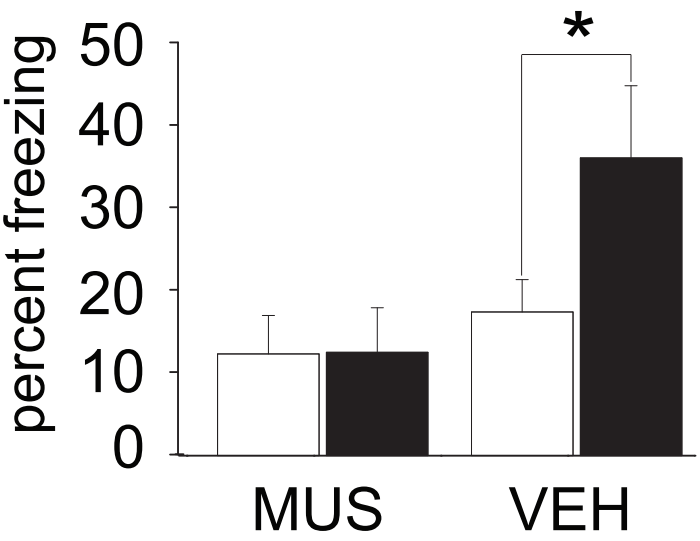
Supplementary Figure 6: Effects of PAG inactivation on unconditioned responding to the US during acquisition. Movement speed (y-axis) during the shock train after infusions of MUS versus VEH into PAG. MUS treated animals exhibited lower URs compared with VEH treated animals (t_{21}= 3.15, p=0.005)



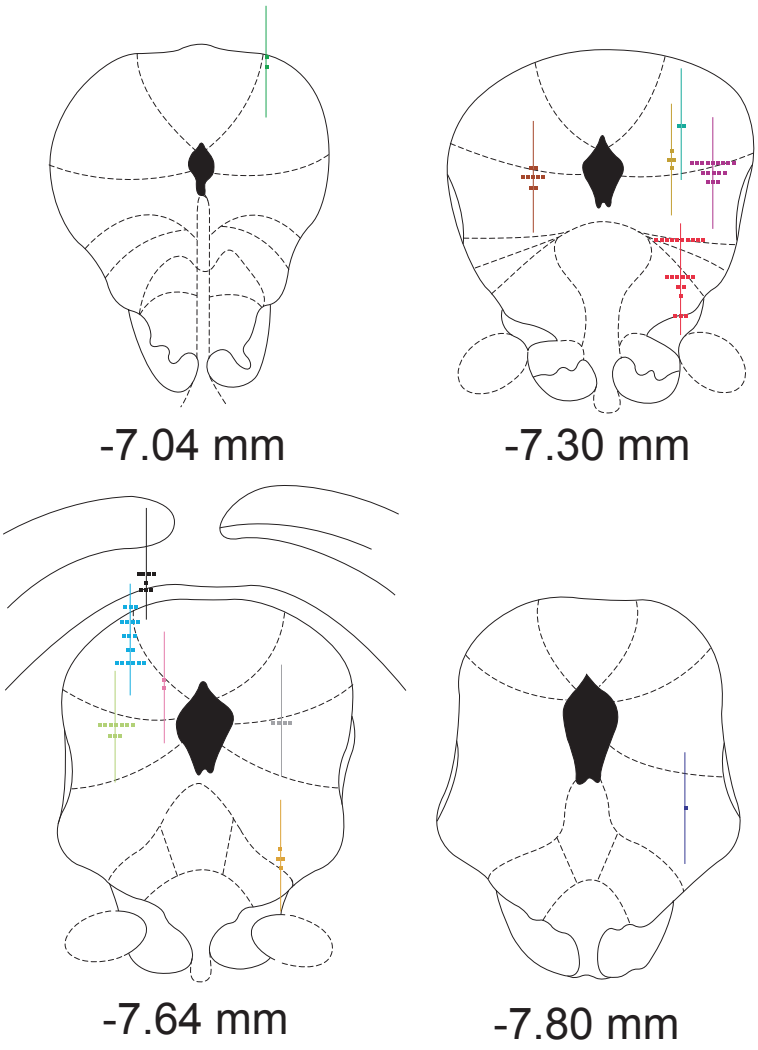
Supplementary figure 7: effect of off-site PAG inactivation on the acquisition of fear conditioning Freezine measured 6 days after conditioning in the off-site control group which had received pre-training infusions of MUS into sites lateral to the PAG. A 2X2 repeated measures ANOVA revealed a main effect ($F_{1,20}=4.417$, $p=0.049$) 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.



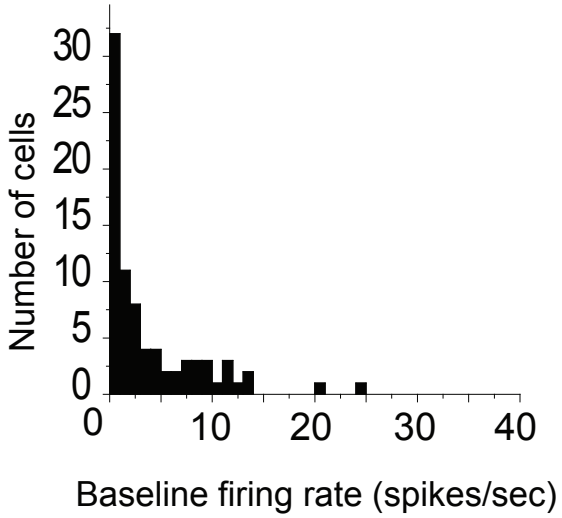
Supplementary Figure 8: Effects of prior PAG inactivation on re-training of fear conditioning Some rats from each group which had been previously fear conditioned (MUS and VEH, see Fig. 4) were given a drug-free retraining session (16 CS-US pairings) immediately after the test trials on the novel platform, 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.



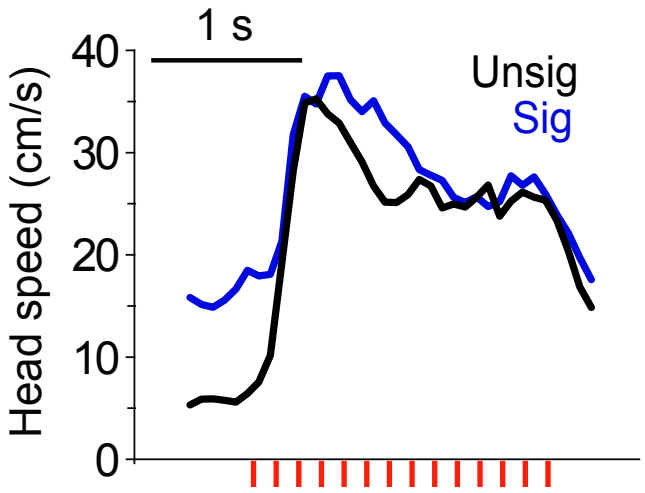
Supplementary Figure 9: Effects of PAG inactivation on expression of previously learned fear conditioning. Some of the rats from the PAG MUS experiments were retrained drug free and infused with either MUS or VEH (as described above) and given another test session. Freezing in well-trained rats during 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.

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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