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Complementary roles for amygdala and periaqueductal gray in temporal-difference fear learning

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Pavlovian fear conditioning is not a unitary process. At the neurobiological level multiple brain regions and neurotransmitters contribute to fear learning. At the behavioral level many variables contribute to fear learning including the physical salience of the events being learned about, the direction and magnitude of predictive error, and the rate at which these are learned about. These experiments used a serial compound conditioning design to determine the roles of basolateral amygdala (BLA) NMDA receptors and ventrolateral midbrain periaqueductal gray (vlPAG) μ-opioid receptors (MOR) in predictive fear learning. Rats received a three-stage design, which arranged for both positive and negative prediction errors producing bidirectional changes in fear learning within the same subjects during the test stage. Intra-BLA infusion of the NR2B receptor antagonist Ifenprodil prevented all learning. In contrast, intra-vlPAG infusion of the MOR antagonist CTAP enhanced learning in response to positive predictive error but impaired learning in response to negative predictive error—a pattern similar to Hebbian learning and an indication that fear learning had been divorced from predictive error. These findings identify complementary but dissociable roles for amygdala NMDA receptors and vlPAG MOR in temporal-difference predictive fear learning.

Significant progress has been made in understanding neural mechanisms for fear learning (LeDoux 2000). This research shows that N-methyl-D-aspartate (NMDA)-receptor-mediated neurotransmission in lateral (LA) and basolateral nucleus of amygdala (BLA) is critical for fear learning (Maren and Quirk 2004). BLA infusions of an NMDA antagonist impair fear acquisition (e.g., Miserendino et al. 1990; Kim et al. 1991) and extinction (e.g., Falls et al. 1992). However, additional amygdala nuclei including central nucleus (e.g., Killcross et al. 1997; Wilensky et al. 2006; Zimmerman et al. 2007) and other structures have also been implicated in fear learning. For example, opioid receptors in the ventrolateral quadrant of the midbrain periaqueductal gray (vlPAG) contribute to fear learning (Fanselow 1998). Systemic or vlPAG μ-opioid receptor (MOR) selective antagonists enhance the acquisition of fear (Young and Fanselow 1992; McNally et al. 2004a) but impair the blocking (McNally and Cole 2006), overexpectation (McNally et al. 2004a), and extinction (McNally et al. 2004b) of fear learning in rats. Systemic administrations of opioid receptor antagonists likewise facilitate acquisition of fear learning in humans (Eippert et al. 2008) but impair the loss of fear caused by exposure-based therapies (Merluzzi et al. 1991; Arntz et al. 1993; Kozak et al. 2007).

A fundamental question concerns how fear learning is distributed among these structures. Of particular interest in the present experiments were the specific roles of vlPAG MOR and BLA NMDA receptors in Pavlovian association formation, given evidence that vlPAG contributes to predictive error during fear learning and the well-established role for BLA in fear learning. A difficulty in answering this question is that fear learning is not a unitary process. Many variables contribute to fear learning, including the physical salience of the events being learned about, the direction and magnitude of predictive error, and so forth. Answering these questions requires isolation of these different variables. The Temporal-Difference (TD) model allows one such approach based on real-time processing of predictive error (Sutton 1988; Sutton and Barto 1990). The TD model has been used to explain learning-related activity in primate midbrain dopamine neurons (e.g., Schultz et al. 1997) and human fMRI BOLD signals (e.g., Seymour et al. 2004), and recently has been employed to examine fear learning (see Cole and McNally 2007a).

A unique assumption of the TD model is that earlier predictors of an outcome are more informative, and so are learned about at the expense of later predictors (Sutton 1988; Sutton and Barto 1990). Therefore, a well-trained CS may undergo a decrement in responding if it is subsequently arranged to precede the US more closely than a second, neutral stimulus, which in turn, should undergo an increment in responding (Sutton and Barto 1981; Kehoe et al. 1987; Jennings and Kirkpatrick 2006). A three-stage blocking-unblocking design exploits this assumption to assess bidirectional changes in fear learning in the same subjects on the same trials (Table 1; Sutton and Barto 1981). In Cole and McNally (2007a) rats received CSA-shock pairings in Stage I. In Stage II they received CSA-CSB-shock pairings that block learning to CSB. In Stage III, a serial overlapping compound, CSB → CSA, was followed by shock. Results demonstrated a within-subjects bidirectional change in responding to the stimuli where responding to CSA decreased while simultaneously fear of CSB increased. According to TD theory, this pattern of responding is the result of two prediction errors during each Stage III trial. The first is a positive prediction error (outcome > prediction) at the onset of CSA, which is due to CSA (a conditioned excitor) possessing reinforcing strength. This second-order learning (Rescorla 1980) results in the unblocking of CSB. The second is a negative prediction error (outcome < prediction) at the onset of the shock US, which is due to the summed strengths of CSA and the recently unblocked CSB exceeding the amount of learning supported by the US (see Sutton and Barto 1990). This overexpectation (Rescorla 1970) causes a loss of fear to both CSA and CSB. Thus, the net change in learning during Stage III is an increment of fear to CSB.
and a decrement of fear to CSA. This within-subjects bidirectional change in fear learning can only be explained by processing of TD predictive error. It cannot be explained by nonassociative processes. Furthermore, this design, involving learning in response to both positive and negative prediction errors in the same subjects during the same trials, is well suited to assessing roles of different brain regions in fear learning.

The experiments reported here studied contributions of BLA NMDA and vIPAG MOR to TD predictive fear learning. We specifically studied the effects of BLA NMDA or vIPAG MOR antagonism on learning in response to the positive and negative prediction error during Stage III of the TD design described above. Our results suggest that these contributions are dissociable but complementary: BLA NMDA receptors determine the rate of learning independent of direction and magnitude of predictive error, whereas vIPAG MOR determine predictive error.

Results

Experiment I: Effects of BLA NR2B receptor antagonism on TD predictive fear learning

Prior research investigating TD prediction errors has omitted Stage II compound training, employing a two-stage design where serial compound conditioning (Stage III reported here) directly follows Stage I (e.g., Kehoe et al. 1987; Jennings and Kirkpatrick 2006). Such a design is useful because TD theory predicts that serial compound training will nevertheless result in loss to CSA and simultaneous gain to CSB. Critically though, the gain to CSB in such a design is de novo acquisition of fear to a novel CS rather than the unblocking of fear to a blocked CS. We adopted the three-stage design in the present experiments for two reasons: First, because it enabled us to study learning about CSs which had been blocked (CSB) or not (CSA) during Stage II. It therefore provides insights into the nature and consequences of blocking (Stage II) and unblocking (Stage III) of fear learning. Second, because Stage II allows control over potential confounding effects of the non-reinforced presentations of CSA and CSB. That is, fear to CSA and CSB was tested daily during Stage III using nonreinforced presentations of each stimulus prior to compound presentation. These test trials provided a measure of performance to each CS unconfounded by the presence of the shock US and the presence of the receptor antagonists. However, it might be suggested that any change in performance during Stage III was simply caused by these test presentations (e.g., extinction) or because these presentations promoted a generalization decrement from Stage II to Stage III (Neely and Wagner 1974). Inclusion of Stage II in the design mitigates against both of these potential confounds.

The aim of Experiment I was to examine the effects of BLA NR2B receptor antagonism on learning in response to the positive and negative prediction errors generated during Stage III. There were three groups: group 0 μg Ifenprodil, group 0.1 μg Ifenprodil, and group 1 μg Ifenprodil. Each group received identical behavioral training during Stages I–III; they differed only in the type of BLA infusion prior to Stage III training (Table 1).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>1 μg</td>
<td>A+</td>
<td>A, B, AB+</td>
<td>A, B, 1 μg Ifenprodil: B → AB+</td>
</tr>
<tr>
<td></td>
<td>0.1 μg</td>
<td>A+</td>
<td>A, B, AB+</td>
<td>A, B, 0.1 μg Ifenprodil: B → AB+</td>
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<td></td>
<td>0 μg</td>
<td>A+</td>
<td>A, B, AB+</td>
<td>A, B, 0 μg Ifenprodil: B → AB+</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>0.5 μg</td>
<td>A+</td>
<td>A, B, AB+</td>
<td>A, B, 0.5 μg CTAP: B → AB+</td>
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<td></td>
<td>0.05 μg</td>
<td>A+</td>
<td>A, B, AB+</td>
<td>A, B, 0.05 μg CTAP: B → AB+</td>
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<td></td>
<td>0 μg</td>
<td>A+</td>
<td>A, B, AB+</td>
<td>A, B, 0 μg CTAP: B → AB+</td>
</tr>
</tbody>
</table>

“*A” was a 30-sec 4-Hz flashing light conditioned stimulus (CS). “B” was a 30-sec 2-B 10-Hz clicker CS. “-” Denotes a 1-sec 0.5-mA foot shock unconditioned stimulus (US). The NR2B receptor antagonist Ifenprodil was microinjected intra-BLA (0.3 μL). The μ-opioid receptor antagonist CTAP was microinjected intra-vIPAG (0.5 μL).

Figure 1A shows mean (± SEM) levels of freezing during the first presentation of CSA on each day of Stage I and during presentation of the AB compound during Stage II. Fear accrued to CSA across Stage I training (F(1,21) = 42.45, P < 0.05), with no significant difference between groups (all F(1,21) < 1, P > 0.05). Levels of pre-CS freezing were low during Stage I (Day 1: Mean = 1%, SEM = 0.5%; Day 2: Mean = 6%, SEM = 1.4%). During Stage II, freezing to the AB compound remained high with no overall significant change in fear across days (F(1,21) < 1, P > 0.05). Levels of pre-CS freezing were low during Stage II (Day 1: Mean = 9%, SEM = 2%; Day 2: Mean = 11%, SEM = 2.2%).

For CSA there was an overall effect of drug (F(1,21) = 7.4, P < 0.05), so that Ifenprodil-treated rats showed significantly more freezing than vehicle-treated rats. There was no overall difference between Ifenprodil doses (F(1,21) < 1, P > 0.05). There was no main effect of trial (F(1,21) = 1.5, P > 0.05). There was a significant two-way interaction between trial and drug versus vehicle (F(1,21) = 8.5, P < 0.05), indicating that the difference between these groups was greater on the second trial. There was no significant two-way interaction between trial and Ifenprodil dose, F(1,11) < 1, P > 0.05. Follow-up tests confirmed that on the first trial all groups showed equivalent levels of freezing (all F(1,11) < 1, P > 0.05), while on the second trial Ifenprodil-treated rats showed significantly more freezing than vehicle-treated rats (F(1,21) = 14.1, P < 0.05), but there was no difference between the two Ifenprodil groups (F(1,21) < 1, P > 0.05). This shows that BLA infusions of the NR2B antagonist prevented the loss of fear to CSA.

For CSB there was no overall effect of drug (F(1,21) = 1.5, P > 0.05). There was an overall effect of Ifenprodil dose (F(1,21) = 8.2, P < 0.05) so that group 1 μg showed significantly less freezing that group 0.1 μg. There was a main effect of trial (F(1,21) = 33.5, P < 0.05), indicating that overall there was significantly more fear on
the second trial. This confirms the unblocking of fear to CSB. There was no significant two-way interaction between trial and drug versus vehicle ($F_{(1,21)} = 1.2, P > 0.05$). However, there was a significant two-way interaction between trial and Ifenprodil dose ($F_{(1,21)} = 9.7, P < 0.05$), indicating that the difference between groups 1 μg and 0.1 μg was greater on the second trial. It might be suggested from inspection of Figure 1B that this interaction was due more to the increase in fear among group 0.1 μg than it was to the absence of such an increase among group 1 μg. Further analysis clarified the effects of Ifenprodil on learning to CSB during Stage III. Specifically, infusions of 1 μg of Ifenprodil prevented fear learning to CSB across Stage III because the difference between group 1 μg and vehicle-treated rats was greater on the second trial than the first trial ($F_{(1,21)} = 6.3, P < 0.05$). This shows that 1 μg Ifenprodil prevented the acquisition of fear to CSB. By contrast, group 0.1 μg and vehicle rats did not differ significantly across days ($F_{(1,21)} < 1, P > 0.05$). This also shows that 0.1 μg Ifenprodil had no significant effect on the acquisition of fear to CSB. Taken together, these results show that BLA infusions of the NR2B antagonist dose dependently prevented the acquisition of fear to CSB.

**Experiment 2: Effects of vlPAG μ-opioid receptor antagonism on TD fear learning**

In this experiment we examined the effects of vlPAG MOR antagonism on learning in response to the Stage III positive and negative prediction errors. There were three groups: group 0 μg CTAP, group 0.05 μg CTAP, and group 0.5 μg CTAP. Each group received identical behavioral training during Stages I–III; they differed only in the type of vlPAG infusion prior to Stage III training (Table 1).

**Histology**

Figure 2C displays the placement of the injector tips in vlPAG. Five animals from group 0.5 μg, one animal from group 0.05 μg, and six animals from group 0 μg were excluded because of incorrect cannula placement. Thus, 24 subjects were included in the analyses (0.5 μg group, $n = 7; 0.05$ μg group, $n = 11; 0$ μg group, $n = 6$).

**Behavior**

**Stages I and II**

Figure 2A shows mean (± SEM) levels of freezing during the first presentation of CSA on each day of Stage I and during presentation of the AB compound during Stage II. Fear accrued to CSA across Stage I training ($F_{(1,21)} = 707, P < 0.05$), with no significant difference between groups (all $F_{(0.5,2.1)} < 3, P > 0.05$). Levels of pre-CS freezing were low during Stage I (Day 1: Mean = 0%, SEM = 0%; Day 2: Mean = 3%, SEM = 1%). During Stage II, freezing to the AB compound remained high with no overall significant change in fear across days ($F_{(1,21)} < 1, P > 0.05$). Levels of pre-CS freezing were low during Stage II (Day 1: Mean = 4%, SEM = 1.1%; Day 2: Mean = 3%, SEM = 1.1%).

**Stage III**

Figure 2B shows mean (± SEM) levels of freezing during drug-free test presentations of CSA and CSB in Stage III. Inspection of these panels suggests that vlPAG CTAP prevented the decrease in fear to CSA and facilitated the acquisition of fear to CSB. Levels of pre-CS freezing were low during Stage III (Day 1: Mean = 2%, SEM = 0.8%; Day 2: Mean = 5%, SEM = 1.9%).

For CSA there was an overall effect of drug ($F_{(1,21)} = 6.6, P < 0.05$), so that CTAP-treated rats showed significantly more freezing than vehicle-treated rats. There was no overall difference between CTAP doses ($F_{(1,21)} < 1, P > 0.05$). There was no main effect of trial ($F_{(1,21)} < 1, P > 0.05$). There was a significant two-way interaction between trial and drug versus vehicle ($F_{(1,21)} = 4.5, P < 0.05$), indicating that the difference between these groups was greater on the second trial. There was no significant two-way interaction between trial and CTAP dose ($F_{(1,21)} = 2.5, P > 0.05$). Follow-up tests confirmed that on the first trial all groups showed equivalent levels of freezing (all $F_{(0.5,2.1)} < 2.1, P > 0.05$), while on the second trial CTAP-treated rats showed significantly more freezing than

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**Figure 1.** Effects of BLA NR2B antagonism on TD fear learning. (A) Mean (± SEM) levels of freezing during the first trial of each day in Stage I (A) and during both the test presentations (A and B) and compound presentations (AB+) across days of Stage II. (B) Mean (± SEM) levels of freezing during Stage III drug-free test presentations of CSA and CSB prior to (Trial 1) and after (Trial 2) serial compound training. Intra-BLA Ifenprodil (0.1 or 1.0 μg) dose dependently prevented both the decrease in fear to CSA and increase to fear CSB shown in the control group (0 μg). (C) Location of microinjection cannula tips in the BLA. Atlas templates were adapted from Paxinos and Watson (1998) (distances in millimeters from bregma).
vehicle-treated rats ($F_{1,21}$ = 10.7, $P < 0.05$), but there was no difference between the two CTAP groups ($F_{1,21} < 1, P > 0.05$). This shows that vPAG infusions of the MOR antagonist prevented the loss of fear to CSA.

For CSB there was no overall effect of drug ($F_{1,21} = 3.7, P > 0.05$) or dose ($F_{1,21} = 3.0, P > 0.05$). There was a main effect of trial ($F_{1,21} = 88.5, P < 0.05$), indicating that overall there was significantly more responding on the second trial. There was no significant two-way interaction between trials and drug versus vehicle ($F_{1,21} < 1, P > 0.05$). However, there was a significant two-way interaction between trials and CTAP dose ($F_{1,21} = 7.2, P < 0.05$), indicating that the difference between groups 0.5 µg and 0.05 µg was greater on the second trial. Follow-up tests confirmed that on the first trial all groups showed equivalent levels of freezing (all $F_{1,21} < 2.1, P > 0.05$), while on the second trial CTAP-treated rats showed significantly more freezing than vehicle-treated rats ($F_{1,21} = 4.3, P = 0.05$). There was also a significant difference between groups 0.5 µg and 0.05 µg ($F_{1,21} = 6.6, P < 0.05$). This shows that vPAG infusions of the MOR antagonist dose dependently facilitated the acquisition of fear to CSB.

Discussion

Animals learn to fear a CS that signals an aversive event as shown by expression of defensive behaviors and autonomic arousal upon subsequent presentations of that CS. Fear learning is not a unitary process. Rather, it is the product of several variables including, but not limited to, the physical salience of the events being learned

There were sufficient numbers of misplaced cannulae to examine the neuroanatomical specificity of vPAG MOR manipulations. Due to the small group size of group 0.05 µg Misplaced ($n = 1$) we combined groups 0.5 µg and 0.05 µg Misplaced into a single CTAP Misplaced group ($n = 6$). One misplaced cannula was located in the dorsal PAG and the remainders were caudal to vPAG. Opioid receptors are distributed throughout the PAG, and we have previously shown that only vPAG opioid receptors regulate fear learning (McNally et al. 2004b). The performance of the CTAP misplaced group during Stage III was compared to group 0 µg Misplaced ($n = 6$). The mean and SEM levels of freezing during Stage III are shown in Table 2. There was significantly more freezing on the first Stage III trial to CSA in group CTAP Misplaced than group 0 µg Misplaced ($F_{1,10} = 7.0, P < 0.05$). It is worth noting that there was no such difference between CTAP and control animals when cannulae were correctly placed in vPAG (see above). Because these significantly elevated levels of freezing confound interpretation we asked whether there was a significant decline in fear to CSA during Stage III. There was ($F_{1,10} = 31.0, P < 0.05$). We then asked whether this decline was greater for group 0 µg Misplaced than group CTAP Misplaced. It was not ($F_{1,10} = 4.0, P > 0.05$). This shows that there was no significant effect on the decline in responding to CSA of misplaced infusions of CTAP. There was no difference between group CTAP Misplaced and group 0 µg Misplaced in responding to CSB on Day 1 of Stage III ($F_{1,10} < 1, P > 0.05$). There was likewise no difference in responding to CSB on Day 2 of Stage III ($F_{1,10} < 1, P > 0.05$). Taken together, these findings are consistent with our prior research (McNally et al. 2004b, 2005; McNally and Cole 2006), and confirm that the effects of CTAP on predictive fear learning were restricted to vPAG.

Table 2. Mean (SEM) performances during Stage III for animals with misplaced cannulae in Experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSA</td>
<td>CTAP</td>
<td>92% (3)</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>76% (5)</td>
</tr>
<tr>
<td>CSB</td>
<td>CTAP</td>
<td>22% (6)</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>20% (5)</td>
</tr>
</tbody>
</table>
about, the direction and magnitude of predictive error, and the rate at which these are learned about. These experiments used a behavioral approach, derived from TD learning rules, allowing isolation of predictive error, to study the roles of BLA NMDA receptors and vPAG MOR in TD predictive fear learning (Sutton and Barto 1981, 1990; Sutton 1988). Rats learned to fear CSA, which signaled shock. In Stage II, CSA was copresented with CSB under conditions designed to block fear learning to CSB. In Stage III, CSB was arranged to precede and then overlap with CSA, the compound being followed by shock. Under these conditions fear of CSA was reduced during Stage III, despite the continued pairing of CSB with shock, whereas fear of CSB was unblocked or increased. This bidirectional change in learning during Stage III is uniquely explained by TD learning rules.

Antagonism of BLA NMDA receptors with the NR2B subunit selective antagonist ifenprodil had pronounced effects on TD predictive fear learning. Learning in response to both the positive and negative prediction errors during Stage III was prevented; fear of CSA and CSB remained unchanged as a consequence of Stage III training. The NR2 antagonist prevented both the increment and decrement in fear otherwise observed during Stage III. This is the first demonstration that BLA NMDA receptors contribute to TD fear learning. The finding that ifenprodil prevented Stage III learning is consistent with past research showing that BLA NR2B antagonism prevents the acquisition (e.g., Rodriguez et al. 2001; Walker and Davis 2008) and extinction (e.g., Sotres-Bayon et al. 2007) of conditioned fear. In the former, fear learning occurs due to a positive prediction error, whereas in the latter fear loss occurs, at least in part, due to a negative prediction error. It is worth emphasizing that the negative prediction error in the present experiments was not produced via omission of the shock US. Instead, it was produced by an overexpectation effect: The summed predictions from CSA and CSB exceeded the amount of learning supported by the shock during Stage III. Importantly, these results show within the same subjects that BLA NR2B antagonism has the same effect on TD learning regardless of the direction of prediction error producing the learning. This finding has important implications for understanding the role of BLA NMDA receptors in fear learning.

The effects of vPAG MOR antagonism on TD predictive learning were distinct from BLA NMDA receptor antagonism and dependent on the direction of the prediction error. vPAG infusions during Stage III enhanced learning in response to the positive (CSB) prediction error but impaired learning in response to the negative (CSA) prediction error. vPAG MOR function there is fear learning, but this learning is divorced by predictive error. Animals that received vPAG CTAP demonstrated a strong increase in responding to CSB with no decrease in responding to CSA. This is the defining feature of Hebbian learning: A CS will undergo increases in excitatory strength when it is paired with a US, but such increases are unconstrained by predictive error, producing effectively limitless increases in fear learning which are immune to extinction. The finding that vPAG CTAP treated rats behaved in a manner anticipated by a Hebbian rule is striking. It highlights the key role for vPAG MOR in enabling predictive error to regulate fear learning. This could occur if vPAG MOR regulated encoding of the predicted outcome (i.e., the weighted average of recent eligible associative strengths). This finding adds to a growing body of evidence that vPAG is critical for regulating fear learning. Traditionally, vPAG has been considered an important output structure coordinating freezing and antinociceptive defensive responses to threatening events (e.g., LeDoux 2000). The present and other findings (Cicala et al. 1990; Fanselow 1998; Seymour et al. 2004; McNally and Cole 2006; Cole and McNally 2007b; Eippert et al. 2008) strongly suggest that the role of vPAG is more complex than simply mediating CR expression. The vPAG has a central role in fear learning because it contributes to regulation of predictive error. In the absence of normal vPAG function, fear learning is unconstrained by prediction error and obeys simpler Hebbian-learning rules.

In the absence of normal BLA NMDA receptor function there was little learning in response to either positive or negative prediction errors. This underscores the obligatory role for BLA NMDA receptors in fear learning. It also shows that normal vPAG function alone is insufficient for predictive fear learning. However, the identical effect of BLA NMDA receptor antagonism on learning in response to positive and negative prediction errors is difficult to reconcile with the fact that the circumstances promoting increments and decrements in fear are different. Increments in fear are produced via positive prediction errors; decrements are produced by negative prediction errors, yet BLA NMDA antagonists impair learning regardless of the direction and magnitude of such errors. A simple and parsimonious explanation is that BLA NMDA receptors act on the product of vPAG-based prediction error to determine the rate at which fear is learned or lost. BLA infusions of an NMDA antagonist prevent increments and decrements in fear because they profoundly reduce the rate of learning produced by CS-US pairings or CS alone presentations.

Finally, it is worth considering some methodological features of these experiments. Responding during Stage III was measured by CS-alone test trials. This was necessary to probe levels of responding uncontaminated by the influence of the shock US or drug microinjections. Such nonreinforced trials could support some extinction to the CSs, but this is unlikely, and the pattern of responding cannot be interpreted in terms of extinction. Neither fear reduction by any extinction might have occurred due to the test trials. First, the small number of these trials following a substantially larger number of conditioning trials renders the possibility of significant extinction remote. Second, there was no such evidence for reductions in responding to CSA during Stage II, which employed the same CS-alone presentations. Third, such an account of the CS-alone trials must also predict low levels of fear to CSB during Stage III because any fear acquired should likewise be extinguished during test. The results demonstrated an increase in fear to CSB. The bidirectional within-subjects change in responding to CSA and CSB during Stage III is uniquely explained by TD learning rules.

We employed unilateral rather than bilateral placements in the BLA and vPAG. We implanted cannula into only one hemisphere for a number of reasons. First, to reduce the possible extent of damage to the PAG and overlying blood vessels, and second, to permit comparison with our previous findings and of the effects of vPAG and BLA manipulations. Finally, previous research has demonstrated that unilateral manipulation of either BLA or vPAG is sufficient to observe a significant effect on learning (e.g., Good and Westbrook 1995; LaBar and LeDoux 1996; McNally and Cole 2006). The results of the present experiment, consistent with this
past work, show that unilateral BLA and vPAG manipulations are sufficient to modulate fear learning.

It is also worth noting that the modalities of the stimuli used were not counterbalanced within the experiments; a visual CS served as CSA, whereas an auditory CS served as CSB. There is little reason to suppose that the pattern of results was due to this. For example, it was suggested that rats are more likely to demonstrate decreased fear to visual CSs and increased fear to auditory CSs. This is highly improbable and inconsistent with the results observed: The visual CS was paired with the shock US during Stages I and II, and freezing reached stable, asymptotic levels across these stages. Freezing to the visual CS only decreased during Stage III under precisely the circumstances (negative prediction error) anticipated by TD theory. According to TD theory, both the visual and auditory CSs undergo decrements during Stage III, it is simply that the auditory CS undergoes an additional increment (due to reinforcement from the already feared CSA) producing a net increase in fear to it. Importantly, both the reduction in freezing to CSA and the increase to CSB were modulated in a coherent manner by BLA infusions of ifenprodil and vPAG infusions of CTAP.

In conclusion, these experiments used a TD design that allows the effects of positive and negative predictive error to be investigated simultaneously as a tool to probe the contributions of the amygdala and midbrain to predictive fear learning. Whereas both BLA NMDA receptors and vPAG MOR are necessary for such learning, neither alone is sufficient for predictive fear learning to occur. BLA NMDA receptors and vPAG MOR make dissociable but complementary contributions to predicting danger. BLA NMDA receptors determine the rate at which fear is acquired or lost, whereas vPAG MOR enable such acquisition and loss to be governed by predictive error.

Materials and Methods

Subjects

Experimentally naïve male Wistar rats (Gore Hill Research Laboratories; Monash Animal Services) were housed in groups of eight in plastic cages. Food and water were freely available. The procedures were in accordance with the New South Wales Government Animal Research Regulation 1995, and were approved by the Animal Care and Ethics Committee of the University of New South Wales.

Apparatus

Conditioning and testing were conducted in a set of four identical chambers (24 cm [length] × 30 cm [width] × 21 cm [height]). The top and rear walls of these chambers as well as the front hinged door were constructed of clear Perspex and the side walls were made of stainless steel. The floor in each chamber consisted of stainless steel rods 4 mm in diameter spaced 15 mm apart (center to center). Each chamber stood 2 cm above a tray of paper pellet bedding (Fibrecycle). The chambers were cleaned with water and the bedding underneath the chambers was changed between rats. These four chambers were located individually within sound-attenuating boxes that were painted white. The boxes were constantly illuminated by infrared LEDs. Ventilation fans provided a constant background noise (67 dB).

CSA was a flashing (4 Hz) presentation of a white fluorescent light producing an illumination level of 75 candela/m² within the chamber. The light was mounted on the ceiling of each sound-attenuating box, immediately above the conditioning chamber. CSB was an 82-dB (A scale) 10-Hz clicker delivered through speakers mounted in the ceiling of each sound-attenuating box. The foot shock US was a 1-sec, 0.5-mA unscrambled AC 50-Hz shock from a constant current generator that was delivered to the rear wall of each box and connected to a digital multiplexer in an adjacent room that, in turn, was connected to a DVD recorder. The stimuli used for conditioning were controlled by computer (LabView, National Instruments).

Surgery and histology

Rats were injected i.p. with a 1.3 mL/kg dose of the anesthetic ketamine at a concentration of 100 mg/mL and with a 0.3 mL/kg dose of the muscle relaxant xylazine at a concentration of 20 mg/mL. Each rat was placed in the stereotaxic apparatus while maintaining the incisor bar at −3.3 mm below horizontal to achieve a flat skull position. A 26-gauge guide cannula (Plastics One) was targeted at either the right basolateral nucleus of the amygdala (BLA; anteroposterior [AP] −2.3 mm, mediolateral [ML] ± 4.9 mm, dorsoventral [DV] −7.7 mm, coordinates relative to bregma) or right ventrolateral midbrain periaqueductual gray (vPAG; AP −0.1 mm, ML ±0.8 mm, DV −5.6 mm coordinates relative to lambda). The guide cannula was fixed in position with dental cement and anchored by jeweler’s screws. A dummy cannula was kept in the guide at all times except during microinjections. Immediately after surgery, rats were injected i.p. with a 0.3-mL dose of a 300 mg/mL solution of procaine penicillin and s.c. with 0.1 mL of a 100 mg/mL cephalzin sodium. Rats were allowed 5 d to recover from surgery, during which time they were handled and weighed daily.

Coronal sections (40 µm) of unfixed tissue were cut, mounted, and stained with cresyl violet to allow verification of cannula placement and assessment of any extraneous damage. The data of any rat were excluded from the statistical analysis if the cannula tip was outside the targeted region or if the region had sustained extensive damage.

Infusions

For infusions, a 33-gauge microinjection cannula was inserted into the guide cannula and connected to a 10-µL glass syringe operated by an infusion pump. The microinjection cannula projected an additional 1 mm ventral to the tip of the guide cannula. For the amygdala, rats were infused with 1 µg, 0.1 µg, or 0 µg of the NMDA NR2B subunit selective antagonist ifenprodil (Sigma-Aldrich) (dissolved in DMSO with 99% Tween 80/pyrogen-free saline) at a total volume of 0.3 µL. For vPAG, rats were infused with 0.5 µg, 0.05 µg, or 0 µg of the MOR selective antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2; Tocris-Cookson) (dissolved in pyrogen-free saline) at a total volume of 0.5 µL. Drugs were infused over a 2-min period, and the microinjection cannula was left in place for an additional 1 min to permit diffusion of the injectate.

Behavioral testing procedures

The design is shown in Table 1. Each experiment had four stages.

Preexposure

Prior to conditioning rats received 2 d of preexposure. Each day, rats received four 30-sec counterbalanced presentations of each of a flashing light (CSA) and a clicker (CSB). Rats were preexposed to the stimuli to facilitate discrimination between them.

Stage I

Days 1 and 2—each day rats received four presentations of CSA coterminating with a 1-sec 0.5-mA foot shock unconditioned stimulus (US). Four to 6 h later all rats received 10-min non-reinforced exposures to the conditioning chambers to reduce levels of contextual fear.

Stage II

Days 3 and 4—each day, after an adaptation period of 180 sec, rats received a single 30-sec nonreinforced presentation of CSA and CSB with a 30-sec ISI. The order of presentations was counterbalanced across days. After another 180-sec period, rats received a single 30-sec reinforced AB compound presentation. Four to 6 h later, all rats received 10-min nonreinforced exposures to the conditioning chambers.
**Stage III**

Days 5 and 6—on day 5, after an adaptation period of 180 sec, rats received a single 30-sec nonreinforced test presentation of CSA and CSB with a 30-sec ITI. The order of test presentations was counterbalanced across days.

After these drug-free test presentations, rats were then removed and infused in the manner described previously then returned to the conditioning chamber. After another 180-sec adaptation period, all rats received an overlapping serial compound of 30 sec CSB and 30 sec CSA coterminating in shock. Four to 6 h later all rats received 10-min nonreinforced exposures to the conditioning chambers. On day 6 all rats received the initial session of test presentations of CSA and CSB.

**Data analysis**

Performance during conditioning and test was recorded. The rats were subsequently scored every 2 sec as either freezing (defined as the absence of all movement other than that required for breathing) or nonfreezing. The number of observations scored as freezing were summed and converted to a percentage. A random sample of the data was scored by a second observer, who was unaware of group allocation. The interrater reliability, that is, the correlation between the percentages of observations each rat was scored as freezing by each observer, exceeded 0.85. The data were analyzed by means of a planned orthogonal contrast testing procedure, and the Type I error rate was controlled at 0.05 for each contrast tested.

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**References**


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