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Opioid Receptors in the Midbrain Periaqueductal Gray Regulate Prediction Errors During Pavlovian Fear Conditioning

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The authors used a within-subject blocking design to study the role of ventrolateral periaqueductal gray (vlPAG) opioid receptors in regulating prediction errors during Pavlovian fear conditioning. In Stage I, the authors trained rats to fear conditioned stimulus (CS) A by pairing it with shock. In Stage II, CSA and CSB were copresented and followed with shock. Two novel stimuli, CSC and CSD, were also copresented and followed with shock in Stage II. CSA blocked fear from accruing to CSB. Blocking was prevented by systemic pretreatment with naloxone. Blocking was also prevented in a dose-dependent and neuroanatomically specific fashion by vlPAG infusions of the μ-opioid receptor antagonist CTAP. These experiments show that vlPAG μ-opioid receptors contribute to Pavlovian fear learning by regulating predictive error.

Keywords: blocking, surprise, predictive learning, PAG, opiod

Exposed to pairings of a conditioned stimulus (CS) with a footshock (unconditioned stimulus [US]), rats learn about the relation between the CS and US. They exhibit this learning in fear reactions including freezing, potentiated startle, and corticosteroid release (Davis, 1992; Fanselow & LeDoux, 1999; Maren, 2001). Fear learning is mediated by glutamatergic neurotransmission in the amygdala. Activation of *N*-methyl-d-aspartate (NMDA) receptors in the amygdala lateral nucleus (LA) detects the CS–US conjunction and initiates signal transduction cascades (e.g., Ca^{2+} and cyclic AMP-dependent signaling) to result in synaptic plasticity and long-term storage of the fear memory (Maren & Quirk, 2004; Schafe, Nader, Blair, & LeDoux, 2001).

The relation captured by amygdala NMDA receptors and synaptic plasticity is temporal contiguity. Temporal contiguity between CS and US inputs to the LA initiates synaptic plasticity and Pavlovian association formation. However, temporal contiguity between CS and US inputs is insufficient for fear conditioning to occur. Temporally contiguous presentations of a CS and footshock can cause fear learning to the CS (e.g., Blanchard & Blanchard, 1969), prevent fear learning to the CS (as in the blocking effect; e.g., Kamin, 1968; McNally, Pigg, & Weidemann, 2004a), or even reduce fear learning to the CS (as in the overexpectation effect; e.g., Kamin & Gaioni, 1974; McNally et al., 2004a; Rescorla, 1970). Rather, the critical requirement for fear learning is the informational relation between the CS and US. This informational relation is carried by the discrepancy between the actual and expected outcomes of the conditioning trial so that only unexpected events and their antecedents are learned about (Dickinson, 1980; Rescorla, 1988). This discrepancy or error of prediction, not CS–US contiguity, initiates Pavlovian association formation (Dickinson, 1980; Rescorla, 1988). The central role for prediction errors in Pavlovian fear learning is demonstrated by the blocking effect. Kamin (1968) subjected rats to pairings of CSA with footshock. Rats then received a compound of CSA and CSB followed by footshock. Kamin's seminal finding was that prior conditioning of CSA blocked fear learning from accruing to CSB. For control rats subjected only to Stage II compound pairings of CSA and CSB with shock, fear learning to CSB proceeded normally. Blocking shows that fear learning is regulated by predictive error: If the error of prediction is large (the shock US is not already predicted by CSA or CSB), a CSB–shock association is formed; if the error is small (CSA already predicts the shock), formation of a CSB–shock association is blocked.

A role for predictive error in regulating Pavlovian fear learning is widely accepted by learning theorists. However, the precise neural mechanisms for predictive error during fear learning remain unclear. Identification of these mechanisms is essential for understanding neural mechanisms of learning (Schultz & Dickinson, 2000). Schull (1979) suggested that endogenous opioids might contribute to predictive error during fear conditioning. Fanselow and colleagues (e.g., Bolles & Fanselow, 1980; Fanselow & Bolles, 1979; Fanselow et al., 1991; Young & Fanselow, 1992) were the first to identify this opioid receptor contribution to fear conditioning with activation of descending pain control circuits. They also provided the first empirical data to bear on this issue. For example, Young and Fanselow (1992) showed that systemic administrations of the opioid receptor antagonist facilitated the acquisition of contextual fear conditioning. Fanselow et al. (1991) showed that intracerebroventricular administrations of CTOP, a μ-opioid receptor antagonist and somatostatin receptor agonist (Chieng, Connor, & Christie, 1996; Connor, Ingram, & Christie, 1997), also facilitated the acquisition of Pavlovian fear condition-

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ing. Fanselow and Bolles (1979) showed that systemic administrations of naloxone facilitated conditioning to a context when it was conditioned in compound with a fear CS. They trained rats to fear a CS by pairing it with shock in context. In Stage II they arranged for the CS to signal a shock in a second context. Rats receiving this Stage II training under naloxone showed greater postshock freezing than did rats receiving Stage II training under saline. However, interpretation of this result is somewhat problematic. For example, Fanselow and Bolles (1979) studied postshock freezing under the influence of naloxone and did not test drug-free rats for fear of the context at longer intervals after conditioning. Therefore, it is unclear whether the differences observed between the naloxone- and saline-treated rats in the postshock period would have been observed in a drug-free test or whether they were long lasting. Moreover, there is considerable controversy surrounding the ability of a discrete CS to block fear conditioning to a context (Williams & LoLordo, 1995).

More recent research has secured a role for endogenous opioids in the associative blocking of fear conditioning. For example, McNally et al. (2004a) trained rats to fear a context by pairing it with shock. They then arranged for an auditory CS to signal shock in that context. Fear conditioning to the auditory CS was blocked by the prior context conditioning. Administration of the opioid receptor antagonist naloxone prior to auditory CS–US pairings prevented this blocking and instated normal conditioning to the auditory CS, as indexed by levels of fear on a drug-free test (see also Matzel, Hallam, & Miller, 1988). McNally et al. (2004a) also showed that administrations of naloxone prevented the overexpectation of Pavlovian fear conditioning. In that experiment, the authors trained rats in Stage I to fear CSA and CSB by pairing the stimuli separately with shock. In Stage II, CSA and CSB were presented in compound and paired with shock. This Stage II training reduced fear of CSA (i.e., there was overexpectation). This overexpectation was prevented by treatment with naloxone prior to Stage II training.

The aim of the present experiments was to study the mechanisms for opioid receptor contributions to associative blocking of fear conditioning. Whereas previous research has shown that endogenous opioids contributed to associative blocking, this research has left unanswered a number of important questions. First, each of the behavioral designs used previously to study opioid receptor contributions to blocking has confounded evidence for blocking with differences in amounts of exposures to the stimuli used during conditioning. Second, the neuroanatomical locus for opioid receptor contributions to blocking is unknown. Finally, the opioid receptor subtype that mediates associative blocking of fear conditioning is unknown. We addressed these questions using a powerful and well-controlled within-subject design.

Experiment 1

In Experiment 1, we used a within-subjects preparation to demonstrate the associative blocking of Pavlovian conditioning. The design is shown in Table 1. In Stage I, we trained rats to fear a visual stimulus, CSA, by pairing it with shock. In Stage II, CSA was presented in compound with a novel auditory stimulus, CSB, and followed with shock. Also occurring during Stage II were pairings of a compound stimulus consisting of a novel visual stimulus, CSC, and a novel auditory stimulus, CSD, with shock.

Table 1
Experimental Design

Experiment	Stage I	Stage II	Test
Experiment 1	A+	AB+, CD+	B vs. D
Experiment 2	A+	Saline: AB+, CD+ Naloxone: AB+, CD+	B vs. D
Experiment 3	A+	Saline: AB+, CD+ CTAP: AB+, CD+	B vs. D
Experiment 4	A+	0 μg CTAP: AB+ 0.025 μg CTAP: AB+ 0.25 μg CTAP: AB+ 2.5 μg CTAP: AB+	B

Note. A and C were 30-s visual conditioned stimuli (CSs; light or flashing light). B and D were 30-s auditory CSs (clicker or noise). CSs were fully counterbalanced. +indicates the 1-s, 0.5-mA footshock unconditioned stimulus. The opioid receptor antagonist naloxone (2.5 mg/kg) was injected subcutaneously. The μ-opioid receptor selective antagonist CTAP was microinjected into ventrolateral periaqueductal gray. In Experiment 3 the CTAP dose was 5 μg. The microinjection volumes were 0.5 μl.

During testing, rats were presented with CSB and CSD separately, and freezing reactions were measured. This design generated different prediction errors for CSB and CSD during Stage II. The prediction error for CSB was low. Prior to Stage II, it had not been paired with shock, and during Stage II it was being conditioned in compound with CSA, which was previously paired with shock. By contrast, the prediction error for CSD was large because neither it nor C had been paired previously with shock. Therefore, we predicted that more learning would accrue to CSD than to CSB during Stage II and that during testing, rats would show more fear of CSD than of CSB. This would be evidence for blocking of fear conditioning to CSB. There are two features of this behavioral design that deserve attention. First, CSB and CSD were both auditory stimuli that were conditioned in compound with visual stimuli CSA and C during Stage II. Second, the critical evidence for blocking was the within-subjects comparison between fear to CSB and CSD. These features mean that differences in test performance between CSB and CSD could have been due only to differences in learning about them during Stage II. They could not be attributed to differences in CS modality, differences in sensitivity to the CS or US, or nonassociative effects of exposures to CS or footshock on expression of fear.

Method

Subjects

The subjects were 16 experimentally naive, adult, male Wistar rats (220–280 g) obtained from a commercial supplier (Gore Hill Research Laboratories, Sydney, Australia). After arrival, rats were housed in groups of 6–8-in. plastic cages maintained on a 12-hr light–dark cycle (lights on at 7 a.m.) and were allowed access to water and food ad libitum. The rats were handled (1–2 min per rat per day) for 3 days prior to surgery to habituate them to the experimenter. The procedures used were approved by the Animal Ethics Committee at the University of New South Wales and were conducted in accordance with the National Institutes of Health's (1986) *Guide for the Care and Use of Laboratory Animals*.

Apparatus

Conditioning and testing were conducted in a set of four identical chambers (24 cm [length] × 30 cm [width] × 21 cm [height]). The front

and rear walls as well as the hinged lid were constructed of clear Perspex, and the end walls were made of stainless steel. The floor 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 (Fibercycle, Mudgeeraba, Australia). The chambers were cleaned with water, and the bedding underneath the chambers was changed between rats. These chambers were located individually within sound-attenuating boxes that were painted white. The boxes were illuminated by a red LED light so that levels of illumination within the conditioning chambers were 15 cd/m^2.

There were two auditory CSs and two visual CSs. An 82-dB (A scale) white noise and an 82-dB (A scale), 20-Hz clicker served as CSB and CSD, respectively, in a fully counterbalanced fashion. These were delivered through speakers mounted in the ceiling of each box. A constant or flashing (8-Hz) presentation of a white fluorescent light producing an illumination level of 75 cd/m^2 within the chambers served as CSA and CSC, respectively, in a fully counterbalanced fashion. The light was mounted on the ceiling of each box, immediately above the conditioning chamber. All CSs were 30 s in duration, and during conditioning they coterminated with the footshock US. This US was a 1-s, 0.5-mA unscrambled AC 50-Hz shock from a constant-current generator that was delivered to the floor of each chamber. The current available to each floor could be adjusted with an in-line milliampere meter. Digital video cameras were mounted on the rear wall of each box and connected to a digital multiplexer in an adjacent room that, in turn, was connected to a videotape recorder. The stimuli used for conditioning were controlled by computer (LabView, National Instruments, Austin, TX).

Procedure

Preexposure. On Day 1, rats were placed in the chambers for 20 min. During this period there were four presentations of each CS in a counterbalanced order. The intertrial interval between CS presentations was 30 s. We preexposed the stimuli to reduce generalization between them in Stage II (for review see Mackintosh & Bennett, 1998).

Stage I. Stage I training occurred on Days 2–4. During 21-min, 10-s sessions, rats received four 30-s presentations of CSA coterminating with footshock. The intertrial interval (ITI) was random, ranging from 60 to 360 s, with a mean of 211 s. Rats remained in the chamber for a further 5 min after final presentation. We exposed rats to the conditioning chamber for 20 min 4–6 hr after each session to reduce the levels of context conditioning.

Stage II. Stage II training occurred on Days 5 and 6. During 21-min, 10-s sessions, rats received two presentations of AB with shock (AB+) and CD+ each day in a fully counterbalanced order. The ITI was random, ranging from 60 s to 360 s with a mean of 211 s. Rats remained in the chamber for a further 5 min after final presentation. We exposed rats to the conditioning chamber for 10 min 4–6 hr after each session to reduce the levels of context conditioning.

Test. Testing took place on Day 7. Rats were placed in the chambers for 14 min. There was a 180-s adaptation period prior to first CS presentation. There were four presentations each of CSB and CSD in a fully counterbalanced order. The ITI between CS presentations was 30 s.

Data Analysis

In all experiments, performance during conditioning and testing was videotaped. The rats were scored every 2 s as either freezing (defined as the absence of all movement other than that required for breathing) or not freezing. The percentage of these observations scored as freezing was then calculated. The videotapes were scored by two observers, one of whom was unaware of group allocation. The interrater reliabilities, that is, the correlation between each observer's ratings of the percentage of observations scored as freezing for each rat, exceeded .85. The data were analyzed by means of a planned orthogonal contrast testing procedure, with a multivariate approach to repeated measures adopted where necessary (O'Brien & Kaiser, 1985). The Type I error rate (α) was controlled at .05 for each contrast tested.

Results and Discussion

The results are shown in Figure 1. The left panel shows mean ($\pm$*SEM*) levels of freezing during the first presentation of CSA on each day of Stage I training. From inspection, it is clear that fear accrued to CSA across the course of training. This is indicated by the significant linear increase in freezing across the 3 days of Stage I training, $F(1, 15) = 169.4$, $p < .05$. The middle panel of Figure 1 shows mean ($\pm$*SEM*) levels of freezing during the first presentations of the AB and CD compounds on each day of Stage II training. From inspection, it is clear that fear was higher to AB than to CD at the start of Stage II training (because CSA had previously been paired with shock) and that fear to CD increased across the course of Stage II training, reaching levels identical to those displayed to the AB compound. These observations were confirmed by the analysis. There was a significant main effect of stimulus type (AB vs. CD), so that there was more freezing to the AB compound than to CD during Stage II, $F(1, 15) = 20.7$, $p < .05$. There was also a main effect of day, so that freezing increased significantly between the first and second day of Stage II, $F(1, 15) = 48.3$, $p < .05$. Finally, there was a significant interaction between these variables, $F(1, 15) = 71.4$, $p < .05$, so that the increase in freezing across Stage II was significantly greater for CD compared with AB. This confirms the acquisition of fear to CD. The right panel of Figure 1 shows mean ($\pm$*SEM*) levels of freezing during test presentations of CSB and CSD. There was more fear to CSD than to CSB, indicating that the presence of the pretrained CSA blocked fear from accruing to CSB during Stage II. The analysis confirmed this evidence for blocking. There was significantly less freezing to CSB than to CSD, $F(1, 15) = 24.4$, $p < .05$. Freezing also decreased significantly in a linear fashion across test, $F(1, 15) = 6.5$, $p < .05$. However the interaction between CS type (B vs. D) and this decrease in freezing was not significant, $F(1, 15) = 0.3$, $p > .05$.

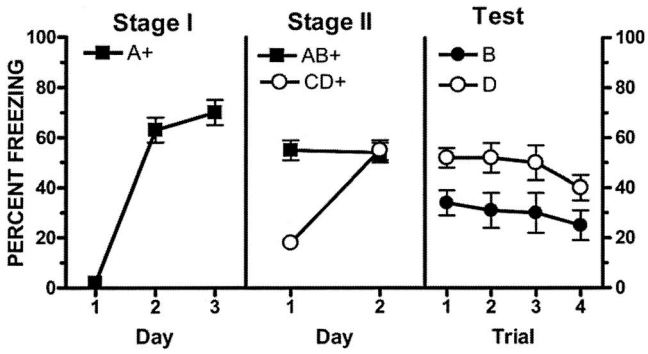

Figure 1. Mean ($\pm$*SEM*) levels of freezing in Experiment 1. A and C were 30-s visual conditioned stimuli (CSs); B and D were 30-s auditory CSs. Freezing developed to A during Stage I (left panel) and to the CD compound during Stage II (middle panel). Performance on testing shows that conditioning of fear to B was less than to D, indicating the presence of blocking. + indicates the presence of footshock with the CS.

Experiment 2

We and others have shown previously that systemic administrations of naloxone prevent the associative blocking of fear conditioning (e.g., Matzel et al., 1988; McNally et al., 2004a). The present experiment differed from this previous work through the use of the within-subjects blocking design to test directly the role of opioid receptors in regulating the associative blocking of Pavlovian fear conditioning. As noted previously, this within-subjects design, unlike that used previously, provides unique control over many nonassociative variables by equating all animals on CS and US presentations. Differences in performance to CSB and CSD during testing can only be due to differences in what is learned about them during Stage II. The design of this experiment is shown in Table 1. The key difference between this experiment and Experiment 1 was the injection of 2.5 mg/kg naloxone prior to each day of Stage II training. The question of interest was whether naloxone would prevent blocking. If so, then fear of CSB and CSD should be similar for naloxone-treated rats, but fear of CSB should be less than fear of CSD for saline-treated rats.

Method

Subjects and Apparatus

Subjects were 16 experimentally naive, adult, male Wistar rats (220–280 g) obtained from the same source and maintained under the same conditions as in Experiment 1. The apparatus was identical to that described for Experiment 1.

Procedure

The procedures for preexposure, Stage I, Stage II, and Test were similar to Experiment 1, but with three exceptions. First, there were 2 days of preexposure rather than 1. Second, rats were injected subcutaneously in the dorsal neck region with 2.5 mg/kg (1 ml/kg) naloxone hydrochloride (Tocris-Cookson, Bristol, England) dissolved in 0.9% (wt/vol) pyrogen-free saline ($n = 8$) or with 1 ml/kg 0.9% (wt/vol) pyrogen-free saline ($n = 8$) 5 min prior to each day of Stage II training. Finally, there was only a single presentation of CSB and CSD during testing.

Results and Discussion

The results are shown in Figure 2. The left panel shows mean ($\pm SEM$) levels of freezing during the first presentation of CSA on each day of Stage I training. Fear conditioning to CSA developed normally across the course of Stage I training. There was a significant linear increase in freezing across the 3 days of Stage I training, $F(1, 15) = 123.1$, $p < .05$. The middle panel shows mean ($\pm SEM$) levels of freezing during the first presentations of the AB and CD compounds on each day of Stage II training. Fear was again higher to AB than to CD at the start of Stage II training, and fear to CD again increased across the course of Stage II training, reaching identical levels to that displayed by the AB compound. Injection of naloxone had little effect on fear conditioning during Stage II. These observations were confirmed by the analysis. There was no main effect of naloxone versus saline during Stage II, $F(1, 14) < 1$, $p > .05$, so that groups showed equivalent levels of freezing. There was no overall significant main effect of stimulus type (AB vs. CD), $F(1, 14) = 2.8$, $p > .05$. There was a main effect of day, so that freezing increased significantly between the first and second day of Stage II, $F(1, 14) = 43.7$, $p < .05$. Finally, there was a significant interaction between day and stimulus type, $F(1, 14) = 40.5$, $p < .05$, so that the increase in freezing across Stage II was significantly greater for CD compared with AB, confirming the acquisition of fear to CD. There were no interactions between the effect of naloxone versus saline and any of these differences during Stage II, all Fs$(1, 14) < 1$, $p > .05$, indicating that the performances during Stage II were identical in the naloxone- and saline-treated animals.

The right panel shows mean ($\pm SEM$) levels of freezing during the test presentation of CSB and CSD. There was more fear to CSD than to CSB among the rats injected with saline prior to Stage II, but levels of fear to CSD and CSB were equivalent for rats

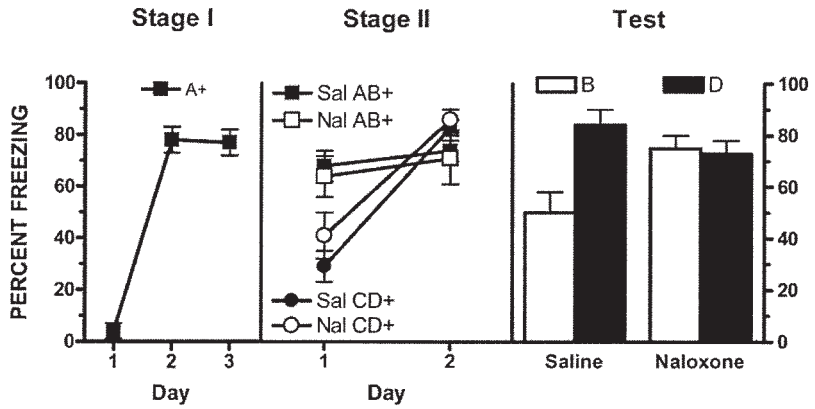

Figure 2. Mean ($\pm SEM$) levels of freezing in Experiment 2. A and C were 30-s visual conditioned stimuli (CSs); B and D were 30-s auditory CSs. Freezing developed to A during Stage I (left panel). Freezing developed normally to the CD compound during Stage II. There was no effect of naloxone during Stage II training (middle panel). Performance during testing shows the presence of blocking (B < D) in rats injected with saline prior to Stage II but not in the rats injected with naloxone in Stage II (B = D). + indicates the presence of footshock with the CS.

injected with naloxone prior to Stage II. The analysis confirmed these observations. There was no main effect of Stage II drug treatment drug (naloxone vs. saline), $F(1, 14) < 1$, $p > .05$. There was a main effect of CS type (B vs. D), $F(1, 14) = 7.6$, $p < .05$, so that there was overall more freezing to CSD than to CSB. Of importance, there was significant interaction between Stage II drug treatment and CS type, $F(1, 14) = 10.4$, $p < .05$, so that the difference between CSB and CSD was significantly greater among saline-treated as compared with naloxone-treated animals, indicating that naloxone prior to Stage II trials prevented the associative blocking of fear. This interpretation was confirmed by analyses of simple effects. The naloxone group showed significantly more fear to CSB than did the saline group, $F(1, 14) = 6.4$, $p < .05$, and these groups did not differ significantly in levels of fear to CSD, $F(1, 14) = 2.3$, $p > .05$.

Experiment 3

The midbrain periaqueductal gray (PAG) is rich in opioid receptors (Gutstein, Mansour, Watson, Akil, & Fields, 1998; Mansour, Fox, Akil, & Watson, 1995; Mansour, Fox, Burke, Akil, & Watson, 1995; Mansour et al., 1994) and has been implicated in many effects of opioid receptor manipulations on behavior (McNally & Akil, 2002), including Pavlovian fear conditioning. For example, Hammer and Kapp (1986) showed that microinjections of the nonselective opioid receptor antagonist naloxone into the PAG potentiated postshock freezing. We have shown that microinjections of naloxone into the ventrolateral quadrant of the PAG (vlPAG) prevent the extinction of Pavlovian fear conditioning (McNally, Pigg, & Weidemann, 2004b). We have also shown that the μ-opioid receptor subtype is especially important for vlPAG opioid receptor contributions to the extinction of Pavlovian fear conditioning, because only infusions of μ-opioid receptor selective antagonists, and not infusions of either κ- or δ-opioid receptor antagonists, into vlPAG impair fear extinction (McNally, Lee, Chiem, & Choi, 2005). The aim of this experiment was to directly test the involvement of vlPAG μ-opioid receptors in regulating prediction errors during the associative blocking of fear conditioning. The design of this experiment is shown in Table 1. The question of interest was whether infusions of the μ-opioid receptor selective antagonist CTAP would prevent associative blocking of Pavlovian fear conditioning. We predicted that if so, then fear of CSB and CSD would be similar for CTAP-treated rats, but fear of CSB would be less than fear of CSD for saline-treated rats.

Method

Subjects and Apparatus

The subjects were 24 experimentally naive, adult, male Wistar rats (220–280 g) obtained from the same source and maintained under the same conditions as Experiment 1. The apparatus was identical to that described for Experiment 1.

Surgery and Histology

Rats were injected intraperitoneally with 1.3 ml/kg of the anesthetic ketamine (Ketapex; Apex Laboratories, Sydney, Australia) at a concentration of 100 mg/ml and with 0.3 ml/kg of the muscle relaxant xylazine (Rompun; Bayer, Sydney, Australia) at a concentration of 20 mg/ml. Each rat was placed in the stereotaxic apparatus (Model 900, Kopf, Tujunga, CA), and the incisor bar was maintained at approximately 3.3 mm below horizontal to achieve a flat skull position. A 26-gauge guide cannula (Plastics One, Roanoke, VA) was implanted into the PAG. The right vlPAG was targeted so that the tip of the guide cannula was positioned 5.6 mm below lambda through a hole drilled 0.1 mm anterior to and 0.8 mm lateral to lambda. We implanted cannula into only one hemisphere to reduce the possible extent of damage to the PAG and overlying blood vessels. We chose the right PAG to facilitate comparison with our previous experiments (McNally et al., 2004b, 2005). The guide cannula was fixed in position with dental cement and anchored with jeweller's screws. A dummy cannula was kept in the guide at all times, except during microinjections. Immediately after surgery, rats were injected intraperitoneally with 0.3 ml of a 300-mg/ml solution of procaine penicillin, subcutaneously with 0.1 ml of a 100-mg/ml cephazolin sodium, and subcutaneously with 5 mg/kg carprofen. Rats were allowed 5 days to recover from surgery, during which time they were handled and weighed daily.

At the conclusion of the experiment, rats were given an overdose of sodium pentobarbital, and their brains were removed. Unfixed brains were sectioned coronally at 40 μm through the PAG by use of a cryostat. Every section through the cannula placements in vlPAG was collected on a glass slide and subsequently stained with cresyl violet. Cannula placements were verified at the microscope by a trained observer who was unaware of the subjects' group designations and used the boundaries defined by Paxinos and Watson (1998). The data of any rat were excluded from the primary statistical analysis if the cannula tip was outside the PAG.

Procedure

Preexposure. The procedure for preexposure was identical to that for Experiment 2.

Stage I. The procedure for Stage I training was similar to that for Experiment 1, with the single exception that rats were placed in white plastic buckets for 5 min prior to each conditioning session. We exposed rats to these buckets because the buckets were later used to house animals during vlPAG infusions in Stage II. These exposures were intended to reduce the novelty of being placed in the buckets during Stage II and therefore reduce potential generalization decrements between Stage I and Stage II, which could interfere with blocking.

Stage II. The procedure for Stage II training was similar to that for Experiment 1, but with the following exceptions. Rats received a 0.5 μL microinjection of 5 μg of the selective μ-opioid receptor antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; Tocris-Cookson, Bristol, England) dissolved in 0.9% (wt/vol) pyrogen-free saline or a 0.5 μL microinjection of 0.9% (wt/vol) pyrogen-free saline. For microinjections, rats were placed in the white plastic buckets, and a 33-gauge microinjection cannula was inserted into the guide cannula. The microinjection cannula projected a further 1 mm ventral to the tip of the guide cannula. The microinjection cannula was connected to a 10-μL glass syringe operated by an infusion pump. Drugs were infused over a 2-min period (0.25 μL/min), and the microinjection cannula was left in place for a further 1 min to permit diffusion of the injectate. All rats were then placed in the conditioning chamber. They received two presentations of AB+ and CD+ in a fully counterbalanced order. These presentations were separated by random ITIs ranging from 60 s to 360 s, with a mean of 211 s. Rats remained in the chamber for a further 5 min after final presentation. We exposed rats to the conditioning chamber for 10 min 4–6 hr after each session to reduce the levels of context conditioning. There was a single day of Stage II training in this experiment so that rats would receive only a single infusion into the vlPAG. Thus, rats received two AB+ conditioning trials and two CD+ conditioning trials in this experiment.

Test. The procedure for testing was identical to that for Experiment 2.

Results and Discussion

Histology

Figure 3A shows the location of the microinjection tips for rats in this experiment. Six rats (3 receiving CTAP and 3 receiving saline) were excluded from the analysis because of misplaced cannulas. The locations of the misplaced cannula for CTAP-treated animals are also indicated. One rat from group saline was also excluded from testing because of infection.

Behavior

The results are shown in Figure 4. The left panel shows mean (±*SEM*) levels of freezing during the first presentation of CSA on each day of Stage I training. Fear conditioning to CSA developed normally across the course of Stage I training. There was a significant linear increase in freezing across the 3 days of Stage I training, $F(1, 17) = 164.5$, $p < .05$. The middle panel shows the mean (±*SEM*) levels of freezing during Stage II. Fear was higher to AB than to CD at the start of Stage II training, and fear to CD increased across the course of Stage II training, reaching identical levels to that displayed toward the AB compound. Microinjection of CTAP into the vlPAG had no effect on fear conditioning during Stage II. The statistical analysis revealed no significant main effect of drug (CTAP vs. saline) during Stage II, $F(1, 16) < 1$, $p > .05$, so that both groups showed equivalent levels of freezing. There was a significant main effect of stimulus type (AB vs. CD) during Stage II, so that there was more freezing to AB than to CD, $F(1, 16) = 9.1$, $p < .05$. There was also a significant main effect of trial, so that freezing increased significantly between the first and second trial of Stage II, $F(1, 16) = 15.01$, $p < .05$. Finally, there was a significant interaction between these variables, $F(1, 16) = 8.9$, $p < .05$, so that the increase in freezing across Stage II was significantly greater for CD compared with AB. This indicates the acquisition of fear to CD. There were no interactions between the effect of CTAP versus saline and any of these differences during-Stage II (all Fs < 2.3, $p > .05$), indicating that performances during Stage II were identical in the CTAP- and saline-treated animals.

The right panel shows the mean (±*SEM*) levels of freezing during the test presentation of CSB and CSD. From inspection, it is apparent that there was more fear to CSD than to CSB among the rats microinjected with saline into the vlPAG prior to Stage II but not among the rats microinjected with CTAP. The analysis confirmed these observations. There was no main effect of Stage II drug treatment (CTAP vs. saline), $F(1, 16) < 1$, $p > .05$. There was a significant main effect of CS type (B vs. D), $F(1, 16) = 6.8$, $p < .05$, so that there was significantly more freezing to CSD than to CSB. Of importance, there was a significant interaction between Stage II drug treatment and CS type, $F(1, 16) = 7.7$, $p < .05$, so that the difference between CSB and CSD was significantly greater among saline-treated as compared with CTAP-treated animals. This shows that CTAP infusions prior to Stage II training selectively prevented blocking of fear to CSB. This interpretation was confirmed by analyses of simple effects. The CTAP group showed significantly more fear to CSB than did the saline group, $F(1, 16) = 7.0$, $p < .05$, but these groups did not differ significantly in levels of fear to CSD, $F(1, 16) = 1.2$, $p > .05$.

At the level of the midbrain studied in this experiment, μ-opioid receptors are expressed most densely in vlPAG (e.g., Gutstein et al., 1998). This fact, combined with the use of relatively small microinjection volumes (0.5 μL), argues strongly against any effect of CTAP at a site distant from microinjection. To confirm neuroanatomical specificity, we examined the performances of animals infused with CTAP but excluded from the original analysis because of misplaced cannulas (see Table 2). One cannula passed into vlPAG but also contacted the aqueduct, one passed through vlPAG to terminate in the deep mesencephalic nucleus, and the last was located in lateral PAG (Figure 3A). Comparison of test performances of the CTAP-treated animals with placements inside versus outside vlPAG provided an important conservative control for the neuroanatomical specificity of the effects of CTAP. The analysis showed significantly greater freezing to CSB among animals microinjected with CTAP into vlPAG, $F(1, 10) = 10.0$, $p < .05$. There was, however, no difference in levels of freezing to CSD between these groups, $F(1, 10) < 1$, $p > .05$. This result confirms the neuroanatomical specificity of vlPAG μ-opioid receptor contributions to blocking of fear conditioning.

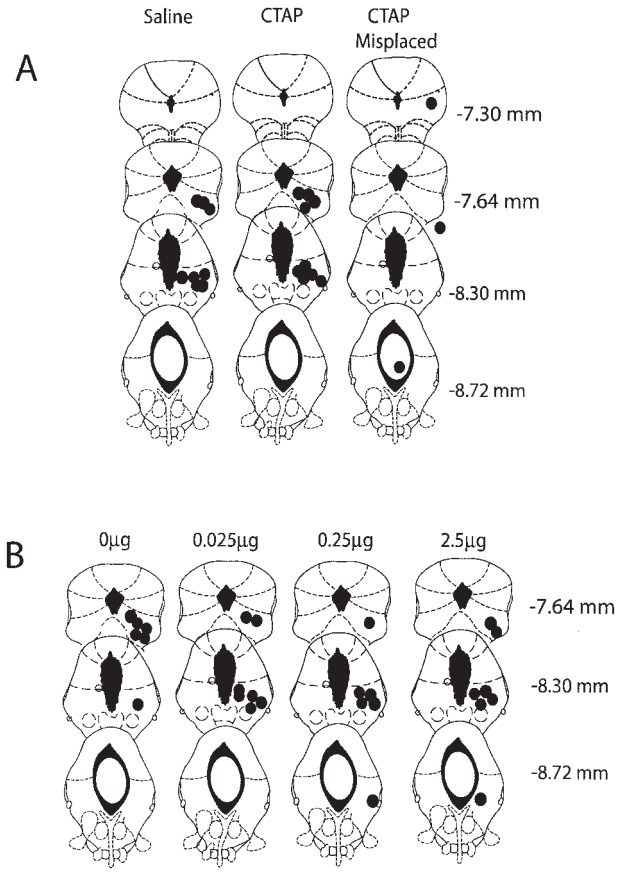

Figure 3. Cannula placement in the periaqueductal gray. Illustration of injection cannula placements for Experiment 3 (A) and Experiment 4 (B) are shown. Placements represented are from all rats included in the final analysis. Distances are in millimeters from bregma. Atlas templates were adapted from Paxinos and Watson (1998). Reprinted from *The Rat Brain in Stereotaxic Coordinates*, G. Paxinos and C. Watson, Figures 49, 50, 53, 54, Copyright 1998, with permission from Elsevier.

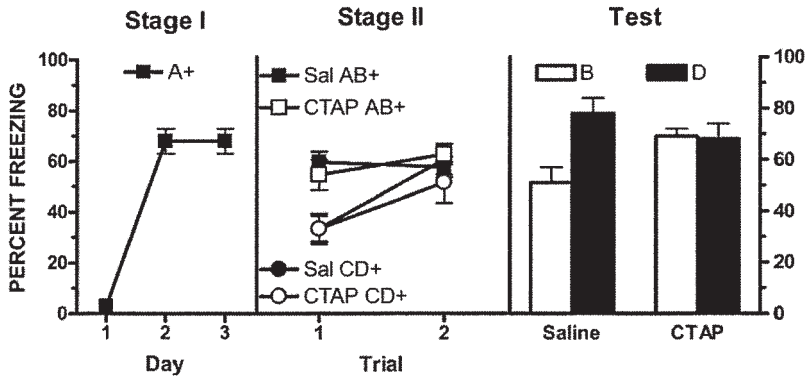

Figure 4. Mean (±*SEM*) levels of freezing in Experiment 3. A and C were 30-s visual conditioned stimuli (CSs); B and D were 30-s auditory CSs. Rats were implanted with cannulas terminating in the ventrolateral quadrant of periaqueductal gray (vlPAG). Freezing developed to A during Stage I (left panel). Freezing developed normally to the CD compound during Stage II. There was no effect of vlPAG microinjections of the μ-opioid receptor antagonist CTAP (5 μg/0.5 μL) during Stage II training (middle panel). Performance during testing shows the presence of blocking (B < D) in rats microinjected with saline prior to Stage II but not in the rats microinjected with CTAP prior to Stage II (B = D). + indicates the presence of footshock with the CS.

Experiment 4

Finally, we assessed whether the amount of fear that accrued to CSB during AB+ trials in Stage II depended on the dose of CTAP infused into vlPAG. The experimental design is shown in Table 1. This design included only groups trained with CSA+ in Stage I and AB+ in Stage II. Rats received infusions (0.5 μL) of 0 μg, 0.025 μg (0.0226 nM) 0.25 μg (0.226 nM), or 2.5 μg (2.26 nM) CTAP prior to Stage II training. We omitted the other CSs (C and D) in this experiment because the results of our previous experiments consistently showed that fear of CSB was less then CSD during testing (i.e., that CSA+ then AB+ training reliably produces blocking of fear conditioning) and that the only effect of opioid receptor antagonism under these circumstances was to prevent the blocking of fear of CSB during Stage II.

Method

Subjects, Apparatus, Surgery, and Histology

The subjects were 32 experimentally naive, adult, male Wistar rats (260–280 g) obtained from the same source and maintained under the same conditions as in Experiment 1. The apparatus was identical to that described for Experiment 1. The procedure for surgery and histology were identical to that for Experiment 3.

Table 2
Mean (SEM) Performances on Test for Animals With Cannula Placements in the Ventrolateral Periaqueductal Gray (v1PAG) and Animals With Misplaced Cannulae in Experiment 3

Location	B	D
vlPAG	69% (3)	68% (7)
Misplaced	49% (6)*	62% (6)[a]

Note. All animals received infusion of 5 μg (0.5 μl) of the μ-opioid receptor antagonist CTAP. There was a significant difference between cannula locations for fear to B but not D.
[a] nonsignificant.
*$p < .05$.

Procedure

The procedure for preexposure, conditioning, and test was similar to that for Experiment 3, but with the following two exceptions. First, only CSA (light) and CSB (noise) were used. Second, the session durations for preexposure, Stage II training, and test were 10 min; 14 min, 50 s; and 7 min, respectively.

Results and Discussion

Histology

Figure 3B shows the location of the microinjection tips for rats in this experiment. Five rats (1 rat in each of groups 0 μg, 0.025 μg, and 2.5 μg, as well as 2 rats in Group 0.25 μg) were excluded from the analysis because of misplaced cannulas.

Behavior

Stage I and Stage II training proceeded normally. The data of primary interest are levels of fear shown to CSB on test. These are shown in Figure 5. The figure shows mean (±*SEM*) levels of freezing during the presentation of CSB on test. Inspection of the figure indicates that fear of CSB increased as a function of the dose of CTAP infused into the vlPAG prior to Stage II. Statistical analysis confirmed a significant linear increase in freezing as dose of CTAP infused into the vlPAG prior to Stage II training increased, $F(1, 22) = 15.1$, $p < .05$.

General Discussion

Pavlovian fear conditioning proceeds as a function of the discrepancy, or predictive error, between the actual and predicted outcomes of a conditioning trial. If the error between the actual and predicted shock US is large, then CS–US associations are formed;

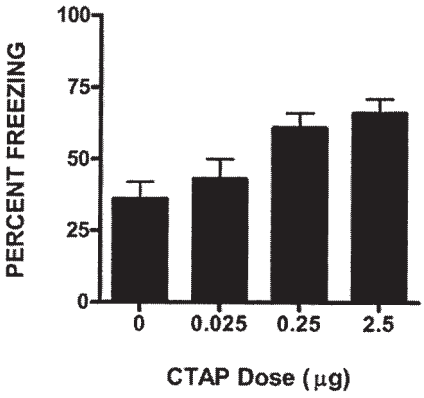

Figure 5. Mean (±*SEM*) levels of freezing during testing in Experiment 4. The behavioral design is shown in Table 1. Rats were implanted with cannulas terminating in the vlPAG. The amount of fear shown to the CS during testing was a linear function of CTAP dose infused into the vlPAG prior to Stage II training.

if the error is small, then formation of CS–US associations is impaired (Dickinson, 1980; Rescorla, 1988). In these experiments, we used a within-subjects blocking design to study the role of opioid receptors in regulating prediction errors during Pavlovian fear conditioning. In Stage I, we trained rats to fear CSA by pairing it with shock. In Stage II, CSA and CSB were copresented, followed by shock. Two novel stimuli, CSC and CSD, were also copresented and followed by shock in Stage II. This design generates different prediction errors for CSB and CSD during Stage II training. The prediction error for CSB was low, because although it had never previously been paired with shock, it was being conditioned in compound with CSA, which was previously paired with shock. By contrast, the prediction error for CSD was large because neither it nor C had been paired previously with shock. Therefore, less learning should accrue to CSB than to CSD during Stage II. During testing there was less conditioned fear to CSB than to CSD, showing that the presence of CSA had blocked fear conditioning to CSB.

Blocking of fear conditioning requires activity at vlPAG μ-opioid receptors. For saline control rats, the predictive error for AB during Stage II was small (CSA already predicted the shock), so formation of a CSB–US association was blocked. By contrast, for rats injected with naloxone or microinjected with CTAP into the vlPAG, although the shock US was already predicted by CSA, and the animals revealed this CSA–shock association through their high levels of fear on the first AB trial of Stage II, the CSB–shock association was not blocked. This prevention of blocking by vlPAG microinjections of CTAP was dose dependent and neuroanatomically specific. It was not due to a general permissive effect of opioid receptor antagonism on fear learning or memory consolidation because neither systemic naloxone nor intra-vlPAG CTAP had any effect on fear conditioning to CSD. Also, prevention of blocking was not due to differences in sensitivity to the shock US, differences in perception of the CSs, alterations in memory processing, or nonspecific effects on fear or expression of the freezing response, because the designs used equated all subjects on exposure to all CSs and the footshock US. Rather, systemic or vlPAG μ-opioid receptor antagonism acted selectively to enable fear learning to CSB. This reveals that naloxone and CTAP prevented the pretrained stimulus CSA from reducing predictive error during Stage II. Thus, vlPAG μ-opioid receptors act specifically during Pavlovian fear conditioning to minimize the discrepancy between the actual and expected outcomes of a CS–US conditioning trial. This is the first evidence directly demonstrating a role for vlPAG μ-opioid receptors in regulating prediction errors during fear learning.

The failure of systemic injections of naloxone and the vlPAG infusions of CTAP to facilitate fear conditioning to the neutral CS, CSD, is worthy of comment. Previous research has shown that systemic naloxone facilitates the acquisition of fear conditioning (e.g., McNally et al., 2004a; Young & Fanselow, 1992). However, there have been no prior published investigations of the effects of vlPAG infusions of opioid receptor antagonists on the acquisition of discrete CS fear conditioning. Hammer and Kapp (1986) reported that vlPAG infusions of naloxone facilitated postshock freezing in a context, but they did not report the performances from a drug-free test at longer intervals after conditioning, nor did they include any controls for nonassociative influences of vlPAG infusions of naloxone. Thus, it is unclear whether any differences observed in the postshock period were associative, whether they were long lasting, or whether they would have been observed in a drug-free test. It is possible that the failure of systemic naloxone and intra-vlPAG CTAP to facilitate fear conditioning to CSD in the present experiments was due to the procedure used. For example, these experiments used fewer CS–US pairings and a more intense US than we have used previously to detect facilitation of conditioning by opioid receptor antagonism (McNally et al., 2004a). However, further research is needed to investigate this issue.

The important finding from these experiments is that vlPAG μ-opioid receptors regulate predictive error during the associative blocking of fear learning. Predictive error can act directly or indirectly on learning. A direct action is achieved by regulating the reinforcing efficacy of the shock US (Rescorla & Wagner, 1972). Blocking of fear conditioning to CSB occurs because the expected shock on AB trials in Stage II is a poorer reinforcer than the surprising shock on CD trials. An indirect action is achieved by the regulation of attention allocated to CSA and CSB during Stage II (Mackintosh, 1975; Pearce & Hall, 1980). Blocking of fear conditioning to CSB occurs as a result of withdrawal of attention from CSB on AB trials because CSA is the better predictor of shock. Our experiments were not designed to permit unequivocal selection between these two different accounts. However, previous investigations into the effects of systemic or intra-vlPAG opioid receptor antagonists on the acquisition (Young & Fanselow, 1992), extinction (McNally et al., 2004a, 2005; McNally & Westbrook, 2003), as well as overexpectation (McNally et al., 2004b) of fear indicate a role for vlPAG opioid receptors in regulating the direct action of prediction errors on fear learning.

McNally and colleagues (McNally, 2005; McNally et al., 2004a, 2005b, 2005) have provided a model of how this direct action might be achieved. According to this model, fear associations are encoded in the amygdala via NMDA receptor-mediated synaptic plasticity. However, these amygdala-based mechanisms for detecting CS–US contiguity are supplemented by a feedback mechanism that contributes to error-correction learning (McLaren, 1989; Schull, 1979). According to this account, the strength of US or

teaching inputs to LA during CS–US pairings is not constant. Rather, an inhibitory feedback signal generated by vlPAG μ-opioid receptors reduces predictive error, or the discrepancy between actual and expected outcomes of a CS–US pairing, by reducing the strength of US inputs to the amygdala. This feedback signal is defined formally as $-\Sigma V$ in the delta rule ($\lambda - \Sigma V$; Rescorla & Wagner, 1972; Sutton & Barto, 1981; Widrow & Hoff, 1960). This feedback signal is behaviorally silent. It is not the same as conditioned analgesia (see below). This signal may regulate US processing in vlPAG, because vlPAG is an important component of ascending sensory pathways (e.g., Keay & Bandler, 2004), or it may regulate this processing at another supraspinal site, for example by gating thalamic processing of the US via extensive vlPAG projections to central and midline thalamus (Krout & Loewy, 2000). Regardless, blocking of fear learning is caused by this inhibitory feedback signal. During Stage II of the experiments reported here, CSA initiated a feedback signal ($-\Sigma V$) in the vlPAG, which reduced the strength of US inputs (λ) to the amygdala. This blocked fear from accruing to CSB.

A central, unique prediction of this model (McNally, 2005; McNally et al., 2004a, 2004b, 2005) is that the same neural mechanisms that mediate the associative blocking of fear also cause the extinction of fear. Extinction refers to the reduction in fear that occurs when a CS that was previously paired with shock is subsequently presented alone. The CS initially elicits fear, but across repeated nonreinforced presentations, the fear is reduced or extinguished. The model states that the same μ-opioid feedback signal ($-\Sigma V$) that causes blocking of fear when the US is present causes the extinction of fear when the US is absent (i.e., $\lambda = 0$). During extinction training, when the CS is presented but the US is omitted, the μ-opioid inhibitory feedback signal causes extinction learning because it is the teaching input to the LA. Indeed, consistent with the claims of this model, fear extinction learning, like associative blocking, depends on opioid receptors (McNally & Westbrook, 2003) and specifically vlPAG μ-opioid receptors (McNally, 2005; McNally et al., 2004b, 2005). This overlap between the neural mechanisms for the associative blocking and extinction of Pavlovian fear conditioning is uniquely consistent with a role for vlPAG μ-opioid receptors in regulating predictive error. It is also interesting because a similar overlap between the neural mechanisms for fear extinction and associative blocking has been observed for Pavlovian appetitive conditioning in primates (e.g., Tobler, Dickinson, & Schultz, 2003; Waelti, Dickinson, & Schultz, 2001) and Pavlovian eyeblink conditioning in rabbits (Kim, Krupa, & Thompson, 1998; Medina, Nores, & Mauk, 2002).

Fanselow and colleagues (Bolles & Fanselow, 1980; Fanselow, 1998; Young & Fanselow, 1992) have also provided a model of opioid receptor regulation of fear conditioning. This model has identified opioid receptor contributions to fear conditioning with their activation of descending analgesic circuits. Presentations of a CS previously paired with shock produce a conditioned analgesia that can depend on μ-opioid receptors in vlPAG (Bellgowan & Helmstetter, 1998). According to this model, blocking occurs because the pretrained CS activates opioid receptor-dependent analgesic circuits, which impair detection of the shock US at the level of the spinal cord dorsal horn (for review see Fanselow, 1998). Naloxone or intra-vlPAG CTAP are held to prevent this conditioned analgesia and therefore increase the pain produced by the shock US during Stage II. The conditioned analgesia model is an important theory of opioid receptor contributions to fear conditioning because it was the first to identify these contributions with endogenous pain control circuits (for review see Bolles & Fanselow, 1980).

The key difference between these models is their view on the nature of the feedback signal, which regulates predictive fear learning. The conditioned analgesia model has identified the feedback signal with variations in the levels of pain produced by the shock US typically used to induce learned fear (Bolles & Fanselow, 1980; Fanselow, 1998), whereas we have rejected this possibility (McNally, 2005; McNally et al., 2004a, 2004b, 2005). There are a number of reasons for divorcing vlPAG μ-opioid receptor contributions to predictive learning from their contributions to pain modulation. For example, these contributions can be dissociated at the intracellular level. μ-opioid receptor activation of descending pain control circuits depends on opioid inhibition of GABAergic neurotransmission within the PAG (Fields, 2004). This GABAergic inhibition is achieved presynaptically by opioid receptor coupling to a voltage-dependent potassium conductance through a pathway involving phospholipase A2, arachidonic acid, and 12-lipoxygenase (Vaughan, Ingram, Connor, & Christie, 1997). By contrast, vlPAG μ-opioid receptor regulation of fear learning is achieved through reductions in cyclic adenosine monophosphate (McNally et al., 2005) and reductions in vlPAG cyclic adenosine monophosphate are unimportant for opioid analgesia (Vaughan et al., 1997). These contributions can also be dissociated at the behavioral level. For example, associative blocking and fear extinction learning share a common vlPAG μ-opioid receptor dependency. However, during extinction training there is no shock US. Thus, vlPAG μ-opioid receptors regulate associative learning in the absence of a shock US. There is also evidence that associative blocking can be dissociated from pain modulation (e.g., Betts, Brandon, & Wagner, 1996). Subjects detect and respond to the shock US during Stage II of the blocking design, and they even learn defensive motor responses to the blocked CS; they simply do not learn to fear that CS (Betts et al., 1996). Together, these findings strongly support the dissociation of opioid receptor contributions to predictive learning from their contributions to pain modulation.

The ability to detect and learn about causal relations between events allows us to use the past to predict the future and to adjust our behavior accordingly. Learning about causal relations depends on what we already know about the events in the relation: If an outcome is unexpected, we learn about cues that predict its occurrence; if the outcome is already expected, information provided by other cues about its occurrence is redundant, and our learning about them is impaired. These experiments show for the first time that μ-opioid receptors in the vlPAG regulate such causal learning during Pavlovian fear conditioning.

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