

The Midbrain Periaqueductal Gray and Fear Extinction: Opioid Receptor Subtype and Roles of Cyclic AMP, Protein Kinase A, and Mitogen-Activated Protein Kinase

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Four experiments studied the opioid receptor subtype and signal transduction mechanisms mediating fear extinction in the ventrolateral quadrant of the midbrain periaqueductal gray (vPAG). Microinjection of a μ - but not a δ - or κ -opioid receptor antagonist into the vPAG retarded extinction. Extinction was also dose-dependently retarded by vPAG infusions of a cyclic AMP (cAMP) analog but was unaffected by infusions of a protein kinase A activator or a mitogen-activated protein kinase inhibitor across wide dose ranges. The results show that fear extinction occurs via activation of vPAG μ -opioid receptors and involves reductions in cAMP. These mechanisms are different from the cellular mechanisms for extinction in the amygdala and from the known cellular mechanisms for opioid analgesia in the vPAG.

Keywords: extinction, fear, opioid, cAMP, error correction

Rats readily learn to fear a conditioned stimulus (CS) that signals the occurrence of an aversive footshock unconditioned stimulus (US). They exhibit this fear in conditioned responses (CRs), including freezing, hypoalgesia, potentiated startle, and increased blood pressure (Davis, 1992; Fendt & Fanselow, 1999; Maren, 2001). Fear learning is mediated, at least in part, by glutamatergic neurotransmission in the amygdala. Specifically, activation of amygdala *N*-methyl-D-aspartate (NMDA) receptors detects the CS–US conjunction and initiates a variety of signal transduction cascades, resulting in synaptic plasticity and long-term association formation (Fanselow & LeDoux, 1999; Maren, 2001).

The fear acquired via CS–US pairings can be reduced or extinguished by exposures to the CS in the absence of the US. A large body of evidence shows that such exposures do not completely erase the CS–US association. Rather, the original association remains largely intact, and a mask is imposed on it (Rescorla, 2001). Recent research has begun to identify the neural mechanisms for extinction learning. Extinction learning depends, at least in part, on amygdala NMDA receptor activation because it can be prevented by intra-amygdala infusions of NMDA receptor antagonists and facilitated by such infusions of NMDA receptor partial agonists (e.g., Falls, Miserendino, & Davis, 1992; Walker, Ressler, Lu, & Davis, 2002). Extinction learning in the amygdala depends also on activation of signal transduction cascades subsequent to NMDA receptor activation, including calcium/calmodulin-dependent pro-

tein kinase, cyclic AMP (cAMP)–dependent protein kinase A (PKA), and the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways, because inhibition of these pathways impairs the development of fear extinction (e.g., Lu, Walker, & Davis, 2001; Szapiro, Vianna, McGaugh, Medina, & Izquierdo, 2003). There is also evidence that fear extinction, as indexed by the freezing CR, depends on the medial prefrontal cortex (mPFC). For example, neurons in the mPFC are activated when an extinguished CS is presented (e.g., Herry & Garcia, 2002), lesions of the mPFC impair long-term retention of extinction learning (e.g., Quirk, Russo, Barron, & Lebron, 2000), and stimulation of the mPFC facilitates the development of fear extinction (e.g., Milad & Quirk, 2002). Together, these findings have been interpreted to mean that amygdala NMDA receptors encode the new learning that occurs during fear extinction and the mPFC contributes to inhibition of the freezing CR as a consequence of this new learning.

We have recently shown that fear extinction also depends on activity at opioid receptors. Systemic administrations of the opioid receptor antagonist naloxone impair fear extinction (McNally & Westbrook, 2003) and related associative error-correction phenomena (e.g., overexpectation; McNally, Pigg, & Weidemann, 2004a). Opioid receptors in the ventrolateral column of the midbrain periaqueductal gray (PAG) appear especially important for this contribution, because infusions of naloxone into the ventrolateral quadrant of the midbrain PAG (vPAG), but not other PAG columns, dose-dependently impair the development of fear extinction (McNally, Pigg, & Weidemann, 2004b). This role for opioid receptors in fear extinction is not attributable to state-dependent learning (McNally et al., 2004b; McNally & Westbrook, 2003). The effects of opioid receptor antagonists on fear extinction are also not due to interference with consolidation of the extinction memory (McNally & Westbrook, 2003). Finally, opioid receptors do not regulate the expression of extinction, because naloxone does not reinstate an already extinguished fear response (McNally et al., 2004b; McNally & Westbrook, 2003). Together, our previ-

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This research was supported by Australian Research Council Grant DP0343808 to Gavan P. McNally.

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ous results show that opioid receptors contribute to the learning that causes fear extinction, and we have specifically suggested that during fear extinction PAG opioid receptors provide an inhibitory signal that amygdala NMDA receptors learn about to cause extinction learning.

The present experiments also study PAG opioid receptor contributions to fear extinction. The first aim was to identify the opioid receptor subtype mediating fear extinction. Molecular cloning and pharmacological studies have identified four receptor subtypes, μ -, δ -, κ -, and nociceptin opioid receptors, and all of these receptors are expressed in the PAG (for a review, see McNally & Akil, 2002; Williams, Christie, & Manzoni, 2001). The second aim was to determine the signal transduction events contributing to fear extinction. Acute opioid receptor activation has diverse cellular consequences, including modulation of potassium and calcium conductances, inhibition of transmitter release, and nuclear signaling (Williams et al., 2001). Of potential relevance to understanding fear extinction are acute opioid receptor inhibition of adenylyl cyclase–cAMP–PKA signaling and acute opioid receptor activation of the ERK/MAPK pathway (Williams et al., 2001). The adenylyl cyclase–cAMP–PKA and ERK/MAPK pathways are key mechanisms for synaptic plasticity (Kandel, 2001; Schafe et al., 2000) and, as reviewed above, have been implicated in fear extinction (e.g., Lu et al., 2001; Szapiro et al., 2003). Opioid receptor modulation of these pathways in the vPAG could therefore be central to fear extinction. To address these aims, we trained rats to fear an auditory CS and then extinguished that fear via nonreinforced presentations of the CS. Prior to extinction training, rats received vPAG infusions of opioid receptor subtype antagonists or modulators of the cAMP, PKA, and ERK/MAPK pathways.

General Method

Subjects

The subjects were 136 experimentally naive, adult male Wistar rats (200–280 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 AM) 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 (1986) guidelines for the care and use of laboratory animals (Publication DHHS National Institutes of Health 86–23).

Apparatus

Conditioning, extinction, and testing were conducted in a set of four identical chambers (24 cm length $\times$ 30 cm width $\times$ 21 cm height). The front and rear walls of these chambers as well as the hinged lid were constructed of clear Perspex, and the end 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 (Fibercycle, Mudgeeraba, Australia). 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 US was a 1-s, 0.8 mA unscrambled V 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 via an in-line milliamperemeter. The CS was a 10-s 74-dB (A scale) 20-Hz clicker delivered through speakers mounted in the ceiling of each box. Video cameras were mounted on the rear wall of each box and connected to a digital multiplexer in an adjacent room, which, in turn, was connected to a video recorder. The stimuli used for conditioning were controlled by computer (LabView, National Instruments, Austin, TX).

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), with the incisor bar maintained at approximately 3.3 mm below horizontal to achieve a flat skull position. A 26-gauge guide cannula (internal diameter = 0.24 mm; external diameter = 0.46 mm; Plastics One, Roanoke, VA) was implanted into the caudal aspect of the right vPAG. The tip of the guide cannula was positioned 5.5 mm below lambda through a hole drilled 0.0 mm anterior to and 0.8 mm lateral to lambda. We implanted cannulas into only one hemisphere to reduce the possible extent of damage to the PAG and overlying blood vessels and to facilitate comparison with our previous work (McNally et al., 2004b). The guide cannula was fixed in position with dental cement and anchored to the skull by jeweller's screws. A dummy cannula was kept in the guide at all times except during microinjections. Immediately after surgery, rats were injected subcutaneously with 5 mg/kg carprofen to provide postoperative pain relief, intraperitoneally with a prophylactic 0.3-ml dose of a 300-mg/ml solution of procaine penicillin, and subcutaneously with 0.1 ml of 100-mg/ml cephazolin sodium. Rats were allowed 5 days to recover from surgery, during which time they were handled and weighed daily.

At the conclusion of the experiments, rats were given an overdose of sodium pentobarbital, and their brain was removed. Unfixed brains were sectioned coronally at 40 μ m through the vPAG via a cryostat. Every fourth section was collected on a glass slide and subsequently stained with cresyl violet. Cannula placements were verified according to the boundaries defined by Paxinos and Watson (1998). The sections were examined under a microscope by a trained observer unaware of the subjects' group designations. The data of any rat were excluded from the statistical analysis if the cannula tip was outside the vPAG.

Drugs and Microinjections

For Experiment 1, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP), nor-binaltorphimine dihydrochloride (Nor-BNI), and Naltrindole hydrogen chloride were obtained from Tocris-Cookson (Bristol, England). All drugs were dissolved in 0.9% (wt/vol) pyrogen-free saline. For Experiment 2, 8-Bromoadenosine 3', 5'-cyclic monophosphate sodium salt (8-Br-cAMP) was obtained from Sigma-Aldrich (Sydney, Australia). It was dissolved in 0.9% (wt/vol) pyrogen-free saline to obtain concentrations of 1 μ g/ μ L, 10 μ g/ μ L, and 20 μ g/ μ L. For Experiment 3, Sp-adenosine 3', 5'-cyclic monophosphorothioate triethylammonium salt (Sp-cAMPS) was obtained from Sigma-Aldrich (Sydney, Australia). It was dissolved in 0.9% (wt/vol) pyrogen-free saline to obtain concentrations of 0.1 μ g/ μ L, 1 μ g/ μ L, 10 μ g/ μ L, and 20 μ g/ μ L. In Experiments 1–3, 0.9% (wt/vol) pyrogen-free saline was used for control microinjections. For Experiment 4, U0126 was obtained from Promega Corporation (Sydney, Australia) and was dissolved in DMSO to obtain concentrations of 0.1 μ g/ μ L, 1 μ g/ μ L, or 5 μ g/ μ L. DMSO was used for control infusions.

All microinjections were a volume of 1 μ L. The use of this relatively large volume was necessary to ensure solubility of the drugs used. It is worth noting that we have previously found that only opioid receptors in

the vPAG contribute to fear extinction because infusions of naloxone into the dorsal or dorsolateral PAG did not affect the development of extinction (McNally et al., 2004b). These findings, coupled with the present location of microinjection cannulas in the caudal PAG where the vPAG is largest, suggest that it is unlikely that any effects of the infusions could be explained by diffusion of the drugs to other PAG columns. For microinjections, a 33-gauge microinjection cannula (internal diameter = 0.10 mm; external diameter = 0.20 mm; Plastics One, Roanoke, VA) was inserted into the guide cannula and connected to a 10- μ L glass syringe (Hamilton, Reno, NV) operated by an infusion pump (World Precision Instruments, Sarasota, FL). The microinjection cannula projected a further 1 mm ventral to the tip of the guide cannula. Drugs were infused over a 3-min period, and the microinjection cannula was left in place for a further 1 min to permit diffusion of the injectate.

Procedure

On Day 0 of the experiment, rats were transported to the laboratory and placed in the conditioning chambers. Five hundred ninety seconds after placement in the chamber, rats received a 10-s presentation of the auditory CS that coterminated with delivery of the US. Rats were removed from the chamber 60 s after the US. Across Days 1–2, rats were transported to the laboratory and microinjected. All rats were placed in the conditioning chamber 5 min after microinjection. Extinction consisted of twelve 30-s presentations of the CS, with presentations spaced 90 s apart. The first CS was presented 120 s after the rat was placed in the chamber. On Day 3, rats were tested. The test consisted of six presentations of the 30-s CS, spaced 90 s apart. The first CS was presented 120 s after the rat's placement in the chamber.

Data Analysis

Performance during conditioning, extinction, and test was videotaped. 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 an observer unaware of group allocation. In all experiments, data were analyzed by means of analysis of variance testing planned contrasts. A multivariate approach to repeated measures was adopted where necessary (O'Brien & Kaiser, 1985). The Type I error rate (α) was controlled at .05 for each contrast tested.

Experiment 1

Experiment 1 studied the opioid receptor subtype that mediates fear extinction. Thirty-two rats were trained to fear an auditory CS via pairing with footshock. The fear that had accrued to that CS was then extinguished over 2 days via 12 presentations of the 30-s CS each day. For the control group (Group Saline [SAL]; $n = 8$), this extinction was preceded by microinjection of saline into the vPAG. For rats in Group CTAP ($n = 8$), extinction was preceded by infusions of 5 μ g of the selective μ -opioid receptor antagonist CTAP. For rats in Group Naltrindole ($n = 8$), extinction was preceded by infusions of 5 μ g of the selective δ -opioid receptor antagonist Naltrindole. For rats in Group Nor-BNI ($n = 8$), extinction was preceded by infusions of 5 μ g of the selective κ -opioid receptor antagonist Nor-BNI. After 2 days of extinction training, rats were tested, in the absence of any antagonists, for their fear reactions to the auditory CS.

Results and Discussion

Histology. The top panel of Figure 1 shows the location of microinjection tips for rats in this experiment. Eleven animals were

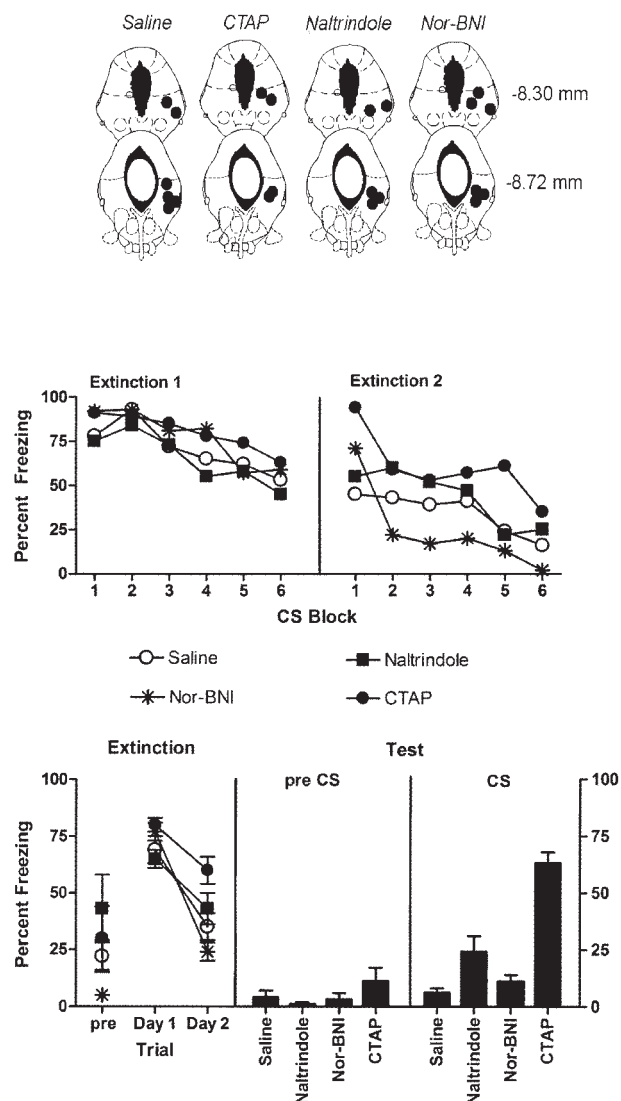


Figure 1. Role of opioid receptor subtypes in the ventrolateral quadrant of the midbrain periaqueductal gray (vPAG) in extinction of conditioned freezing. Top panel: Location of injection cannula placements in the vPAG (distances in millimeters from bregma). Reprinted from *The Rat Brain in Stereotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, Figures 53 and 54, Copyright 1998, with permission from Elsevier. Middle panel: Mean ($\pm$ SEM) percentage of freezing per block of two conditioned stimuli (CSs) across the 2 days of extinction training for control rats (which received saline) and rats microinjected with μ -opioid receptor antagonist (5 μ g D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ [CTAP]), a δ -opioid receptor antagonist (5 μ g Naltrindole), or a κ -opioid receptor antagonist (5 μ g nor-binaltorphimine dihydrochloride [Nor-BNI]). Bottom panel: Mean ($\pm$ SEM) percentage of freezing during the first 2 min prior to conditioned stimulus onset on Day 1 and average freezing across the 2 days of extinction training as well as freezing during the drug-free test for the four groups described above.

excluded from the experiment because of incorrect cannula placement. Thus, 21 animals were included in the analyses (Group SAL, $n = 6$; Group CTAP, $n = 4$; Group Naltrindole, $n = 5$; and Group Nor-BNI, $n = 6$).

Behavior. The middle panel of Figure 1 shows the mean levels of freezing during the 2 days of extinction training, averaged across blocks of two CS presentations. The bottom left panel of Figure 1 shows the average levels of freezing per day for each day of extinction training as well as during the 2 min immediately prior to the first CS on Day 1. The bottom middle panel of Figure 1 shows the levels of freezing in the 2 min immediately prior to the first CS on test. The bottom right-hand panel of Figure 1 shows the average level of CS freezing on test. Inspection of the figure indicates that, overall, there is evidence for extinction of conditioned fear. Averaged across groups, levels of freezing decreased significantly between Day 1 and Day 2 of extinction training, $F(1, 17) = 61.9$, $p < .01$, and freezing was low on test. The statistical analysis of Day 1 pre-CS levels of freezing failed to detect any significant differences between Group SAL and Group CTAP, $F(1, 17) < 1$, $p > .05$; between Group SAL and Group Naltrindole, $F(1, 17) = 1.2$, $p > .05$; or between Group SAL and Group Nor-BNI, $F(1, 17) < 1$, $p > .05$. The analysis of test day pre-CS freezing also failed to detect any significant differences between Group SAL and Group CTAP, $F(1, 17) = 1.5$, $p > .05$; between Group SAL and Group Naltrindole, $F(1, 17) < 1$, $p > .05$; or between Group SAL and Group Nor-BNI, $F(1, 17) < 1$, $p > .05$.

Fear extinction was retarded by vIPAG infusions of the μ -opioid receptor antagonist CTAP. During extinction training there were significantly higher levels of freezing in Group CTAP as compared with Group SAL, $F(1, 17) = 4.9$, $p < .05$. This difference did not interact with the decrease in freezing observed across the 2 days of extinction training, $F(1, 17) = 1.3$, $p > .05$. It is important to note that there was also a significant difference between Group SAL and Group CTAP on the drug-free test, $F(1, 17) = 60.4$, $p < .01$, indicating that the differences between groups were preserved on that test. These results identify the μ -opioid receptor as an important opioid receptor subtype for fear extinction.

This role for μ -opioid receptors in fear extinction was neuro-anatomically specific to the vIPAG. We have previously shown that infusions of naloxone into the dorsal or dorsolateral PAG do not impair the development of fear extinction. We compared the test performance of animals in the CTAP group excluded from the analysis because of cannula placements outside the vIPAG with the performance of those animals included in the analysis because of cannula placements in the vIPAG (see Figure 2). Three animals in Group CTAP had incorrect cannula placement (an additional animal in Group CTAP was not extinguished or tested because it developed an infection). Inspection of the figure confirms that CTAP impaired extinction only when it was infused into the vIPAG. The analysis shows that, overall, the rats in Group vIPAG displayed significantly more freezing during extinction training than rats in Group Misplaced, $F(1, 5) = 6.6$, $p = .05$. It is important to note that there was also a significant difference among groups in CS freezing on test, $F(1, 5) = 15.4$, $p = .01$. Thus, only infusions of CTAP into the vIPAG impaired extinction.

There is no evidence that extinction was retarded by the κ -opioid receptor antagonist Nor-BNI. There were no differences in levels of freezing between Group Nor-BNI and Group SAL during extinction training, $F(1, 17) < 1$, $p > .05$; no interaction between these group differences and the decrease in freezing observed across the 2 days of extinction training, $F(1, 17) = 3.1$, $p > .05$; and no difference between these groups on test, $F(1, 17) < 1$, $p > .05$.

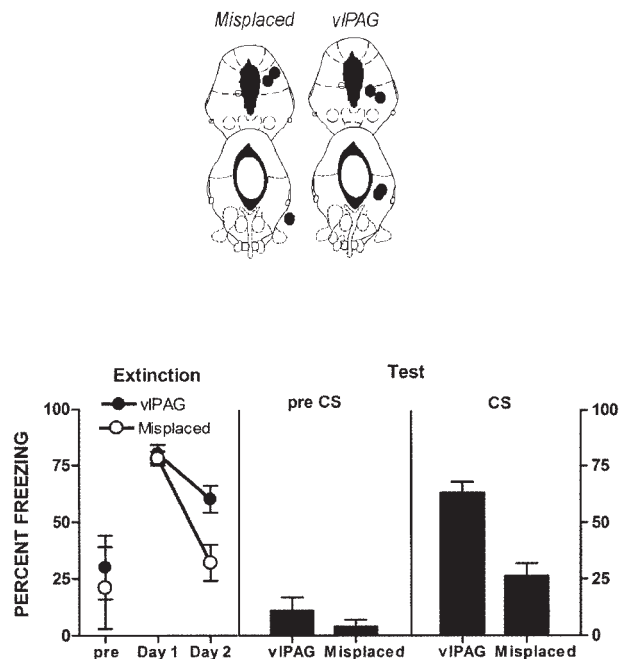


Figure 2. A comparison of the effects of the μ -opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂ (CTAP) infused into or outside the ventrolateral quadrant of the midbrain periaqueductal gray (vIPAG) on fear extinction. Top panel: Location of injection cannula placements in the vIPAG (distances in millimeters from bregma). Reprinted from *The Rat Brain in Stereotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, Copyright 1998, with permission from Elsevier. Bottom panel: Mean ($\pm$ SEM) percentage of freezing per block of two conditioned stimuli (CSs) across the 2 days of extinction training for rats microinjected with μ -opioid receptor antagonist CTAP into the vIPAG (Group vIPAG) and rats microinjected with CTAP but excluded from the original analysis (see Figure 1) because of misplaced cannulas (Group Misplaced).

There is some evidence that fear extinction involved the δ -opioid receptor, because although there was neither a difference in levels of freezing between Group Naltrindole and Group SAL during extinction training, $F(1, 17) < 1$, $p > .05$, nor an interaction between these group differences and the decrease in freezing observed across the 2 days of extinction training, $F(1, 17) = 1.2$, $p > .05$, there was a significant difference between these groups on test, $F(1, 17) = 6.7$, $p < .05$. The fact that Group Naltrindole did not significantly differ from Group SAL during extinction training but did differ from Group SAL on test raises the possibility that there was a generalization decrement between extinction training and test in this group. In other words, there was no evidence during extinction training that Naltrindole impaired extinction, but there was some evidence from test that extinction training under Naltrindole was less effective than extinction training under saline. However, this test was conducted drug free. The most reasonable interpretation is that the small loss of extinction between training and test was due to state-dependent learning. Some portion of the extinction that had accrued under Naltrindole failed to generalize to the test in a drug-free state. This same line of reasoning cannot be applied to the results with the μ -antagonist CTAP because there was a significant effect of CTAP during extinction training under

the drug and on the drug-free test. There was no evidence for state-dependent learning with CTAP.

It might be suggested that the infusions of CTAP were aversive and that they induced fear on their own or simply inflated fear of the CS. There are three difficulties with these suggestions. First, there was no evidence here (e.g., pre-CS freezing on Day 1) that CTAP produced or increased fear, and it has previously been shown that systemic or intra-vlPAG opioid antagonists do not elicit freezing in nonshocked animals (Hammer & Kapp, 1986; McNally & Westbrook, 2003). Second, impaired extinction was apparent during extinction (under CTAP) and during test (in the absence of CTAP). Finally, associative learning theory shows that manipulations that increase fear of a CS facilitate, not impair, fear extinction (conversely, manipulations that reduce fear of a CS impair fear extinction). There is an abundance of evidence from a variety of species, including rats and humans, supporting this relation (e.g., Lovibond, Davis, & O'Flaherty, 2000; Rescorla, 2000, 2003; Rescorla & Wagner, 1972; Thomas & Ayres, 2004; Wagner, 1969; Wagner & Rescorla, 1972). Manipulations that increase the fear elicited by the CS increase the discrepancy between the actual and the expected outcome of the extinction trial and facilitate extinction. Neither the present results nor our previous results can be explained by the supposition that the infusions increased fear of the CS.

Experiment 2

Acute activation of opioid receptors can inhibit adenylyl cyclase and decrease intracellular cAMP (Williams et al., 2001). Therefore, we studied whether an inhibition of PAG adenylyl cyclase–cAMP was causal to fear extinction. If opioid receptor activity in the vlPAG causes fear extinction by inhibiting PAG adenylyl cyclase–cAMP, then extinction should be prevented by a manipulation that increases intracellular cAMP. The present experiment studies this possibility.

Thirty-two rats were trained to fear an auditory CS via pairing with footshock. The fear that had accrued to that CS was then extinguished. For the control group, Group 0 μg ($n = 8$), this extinction was preceded by microinjection of saline into the vlPAG. For the experimental groups, extinction was preceded by infusions of the membrane-permeable cAMP analog 8-Br-cAMP. The doses used were 1 μg (Group 1 μg ; $n = 8$), 10 μg (Group 10 μg ; $n = 8$), and 20 μg (Group 20 μg ; $n = 8$). After 2 days of extinction training rats were tested, in the absence of 8-Br-cAMP, for fear reactions to the auditory CS.

Results and Discussion

Histology. The top panel of Figure 3 shows the location of microinjection tips for rats in this experiment. Nine animals were excluded from the experiment because of incorrect cannula placement. Thus, 23 animals were included in the analyses (Group 0 μg , $n = 6$; Group 1 μg , $n = 5$; Group 10 μg , $n = 5$; Group 20 μg , $n = 7$).

Behavior. The results from extinction and test are shown in the middle and bottom panels of Figure 3. The format for this figure is the same as described for Figure 1. Inspection of the figure indicates that, overall, there is evidence for extinction of conditioned fear. The statistical analysis of Day 1 pre-CS levels of

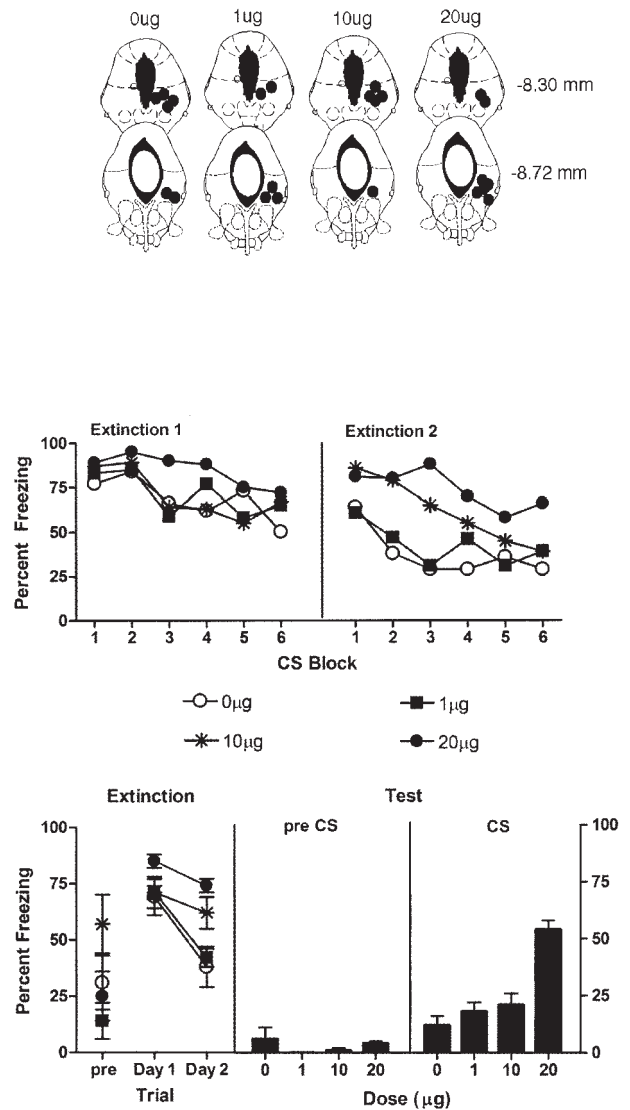


Figure 3. Role of reduced ventrolateral quadrant of the midbrain periaqueductal gray (vlPAG) cyclic AMP (cAMP) in extinction of conditioned freezing. Top panel: Location of injection cannula placements in the vlPAG (distances in millimeters from Bregma). Reprinted from *The Rat Brain in Stereotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, Copyright 1998, with permission from Elsevier. Middle panel: Mean ($\pm$ SEM) percentage of freezing per block of two conditioned stimuli (CSs) across the 2 days of extinction training for control rats (Group 0 μg) and rats microinjected with 1 μg , 10 μg , or 20 μg of the membrane-permeable cAMP analog 8-Bromoadenosine 3', 5'-cyclic monophosphate sodium salt. Bottom panel: Mean ($\pm$ SEM) percentage of freezing during the first 2 min prior to CS onset on Day 1 and average freezing across the 2 days of extinction training as well as freezing during the drug-free test for the four groups described above.

freezing confirmed that there were no differences among groups in pre-CS freezing, $F(1, 19) < 1$, $p > .05$. The statistical analysis of test day pre-CS freezing likewise failed to detect significant differences in levels of pre-CS freezing, $F(1, 19) < 1$, $p > .05$. Averaged across groups, levels of freezing decreased significantly

between Day 1 and Day 2 of extinction training, $F(1, 19) = 34.5$, $p < .05$, and freezing was low on test. This extinction depended on reductions in vIPAG cAMP because it was retarded, in a dose-dependent manner, by infusions of 8-Br-cAMP. Levels of freezing during extinction were a linear function of 8-Br-cAMP dose, $F(1, 19) = 11.9$, $p < .05$, so that as dose increased, so did levels of freezing. This linear trend interacted with the decrease in freezing observed across the 2 days of extinction training, $F(1, 19) = 7.3$, $p < .05$, so that as dose of 8-Br-cAMP increased, the amount of extinction decreased in a linear fashion. Finally, these differences were preserved on the drug-free test, so that freezing on a test was a linear function of dose of 8-Br-cAMP, $F(1, 19) = 37.9$, $p < .05$, which shows that vIPAG infusions of 8-Br-cAMP dose-dependently impaired the development of fear extinction. This impairment of fear extinction by 8-Br-cAMP cannot be attributed to a failure of any extinction learning that occurred under the drugs to generalize to the drug-free test (e.g., state-dependent learning), because impaired extinction was observed during extinction training under the drugs and also on the drug-free test.

It is worth emphasizing that one cannot explain these results by supposing that the infusions of 8-Br-cAMP produced or increased fear. Inspection of the levels of fear during the pre-CS period on Day 1 of extinction training indicates that, of the groups infused with 8-Br-cAMP, only the 10- μ g group (average 57% of observations scored as freezing) appeared to show levels of fear different from those observed in the controls (average 31% of observations scored as freezing). In interpreting this difference, it is important to note that (a) this dose of the drug did not impair the development of fear extinction, (b) the standard deviations for both these groups were large (Group 0 μ g, $SD = 31$; Group 10 μ g, $SD = 32$), and (c) this difference was not statistically significant.

Experiment 3

Increases in intracellular cAMP can lead to the phosphorylation and activation of the cAMP-dependent PKA. Given that acute activation of opioid receptors inhibits this signaling pathway and that reductions in vIPAG cAMP are critical for fear extinction, we studied whether reductions in vIPAG PKA were also causal to fear extinction. If opioid receptor activity in the vIPAG causes fear extinction by inhibiting PKA, then extinction should be prevented by manipulations that increase vIPAG PKA. The present experiment studied this possibility.

Forty rats were trained to fear an auditory CS via pairing with footshock. The fear that had accrued to that CS was then extinguished. For the control group, Group 0 μ g ($n = 8$), extinction was preceded by microinjection of saline into the vIPAG. For the experimental groups, extinction was preceded by infusions of Sp-cAMPS. Sp-cAMPS is a potent, membrane-permeable activator of PKA. The doses used were 0.1 μ g (Group 0.1 μ g; $n = 8$), 1 μ g (Group 1 μ g; $n = 8$), 10 μ g (Group 10 μ g; $n = 8$), and 20 μ g (Group 20 μ g; $n = 8$). These doses were chosen to cover as wide a dose range as possible without producing observable behavioral and autonomic alterations. Pilot studies revealed that higher doses (30 μ g and higher) produced profound alterations that could have interfered with the freezing CR (see also Punch, Self, Nestler, & Taylor, 1997). After 2 days of extinction training, rats were tested, in the absence of Sp-cAMPS, for fear reactions to the auditory CS.

Results and Discussion

Histology. The top panel of Figure 4 shows the location of microinjection tips for rats in this experiment. Nine animals were excluded from the experiment because of incorrect cannula placement. Thus, 31 animals were included in the analyses (Group 0 μ g,

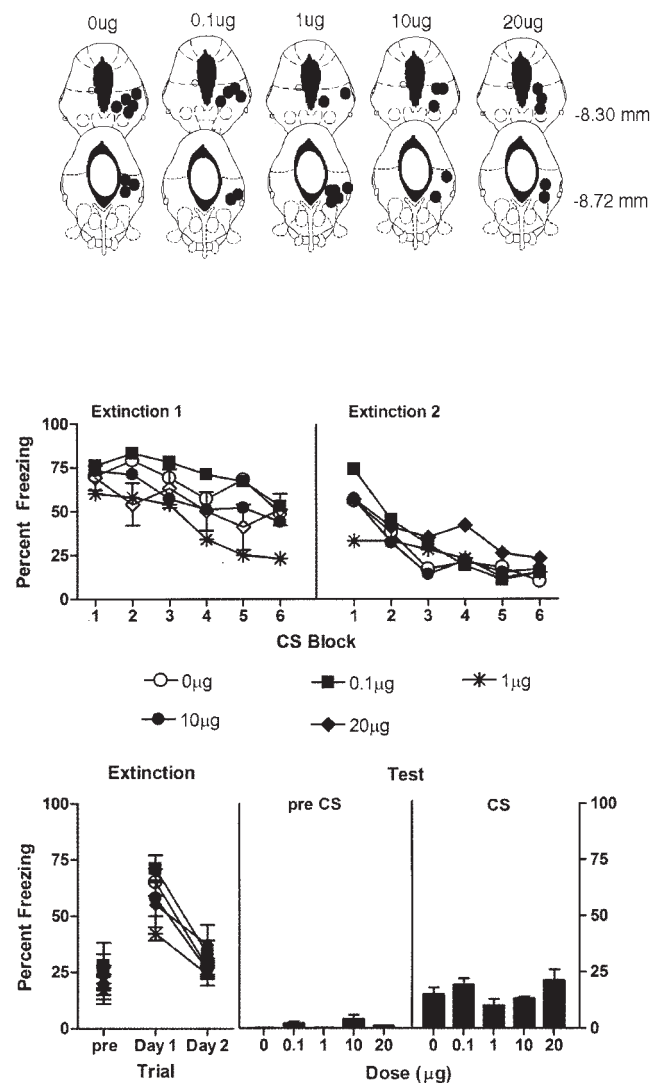


Figure 4. Role of reduced ventrolateral quadrant of the midbrain periaqueductal gray (vIPAG) protein kinase A (PKA) in extinction of conditioned freezing. Top panel: Location of injection cannula placements in the vIPAG (distances in millimeters from bregma). Reprinted from *The Rat Brain in Stereotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, Copyright 1998, with permission from Elsevier. Middle panel: Mean ($\pm$ SEM) percentage of freezing per block of two conditioned stimuli (CSs) across the 2 days of extinction training for control rats (Group 0 μ g) and rats microinjected with 0.1 μ g, 1 μ g, 10 μ g, or 20 μ g of the PKA activator Sp-adenosine 3', 5'-cyclic monophosphorothioate triethylammonium salt. Bottom panel: Mean ($\pm$ SEM) percentage of freezing during the first 2 min prior to CS onset on Day 1 and average freezing across the 2 days of extinction training as well as freezing during the drug-free test for the five groups described above.

$n = 8$; Group 0.1 μg , $n = 6$; Group 1 μg , $n = 7$; Group 10 μg , $n = 5$; Group 20 μg , $n = 5$).

Behavior. The results from extinction and test are shown in the middle and bottom panels of Figure 4. The format for this figure is the same as described for Figure 1. Inspection of the figure indicates that, overall, there is evidence for extinction of conditioned fear. There were no differences among groups in pre-CS levels of freezing either on Day 1, $F(1, 26) < 1$, $p > .05$, or at test, $F(1, 26) < 1$, $p > .05$. Averaged across groups, levels of freezing decreased significantly between Day 1 and Day 2 of extinction training, $F(1, 26) = 67.2$, $p < .05$, and freezing was low on test. This extinction was unaffected across a wide dose range by vPAG infusions of Sp-cAMPS. There was no difference among groups in freezing during extinction training as a linear function of Sp-cAMPS dose, $F(1, 26) < 1$, $p > .05$. There was no interaction between group differences and the decrease in freezing across extinction training, $F(1, 26) = 3.9$, $p > .05$. Finally, there were no differences among groups in the amount of freezing on test as a linear function of Sp-cAMPS dose, $F(1, 26) < 1$, $p > .05$. In other words, there is no evidence from this experiment that reductions in vPAG PKA mediate fear extinction, because infusions of Sp-cAMPS did not impair the development of fear extinction.

Experiment 4

ERK/MAPK signaling plays an important role in synaptic plasticity and associative learning. Amygdala infusions of MAP kinase kinase (MAPKK/MEK) inhibitors impair long-term potentiation and acquisition of fear conditioning (e.g., Schafe et al., 2000). Critically, these infusions also impair the development of fear extinction (Lu et al., 2001). Activation of ERK/MAPK has therefore been accorded an important role in fear extinction (for a review, see Davis, 2002). Acute opioid receptor activation can increase ERK/MAPK activity in vitro (e.g., Fukuda, Kato, Morikawa, Shoda, & Mori, 1996; Kramer & Simon, 2000; Li & Chang, 1996) and in vivo (e.g., Eitan et al., 2003; Ozaki, Narita, Ozaki, Khotib, & Suzuki, 2004) via a number of pathways, including phosphatidylinositol 3-kinase (Ai, Gong, & Yu, 1999) and receptor internalization (e.g., Ignatova, Belcheva, Bohn, Neuman, & Coscia, 1999). These findings raise the possibility that vPAG μ -opioid receptor activation of ERK/MAPK contributes to fear extinction. Although our previous experiments show that extinction involves reductions in vPAG cAMP, this does not exclude a potential involvement for the ERK/MAPK cascade. μ -opioid receptor activation of MAPK can be independent of cAMP/PKA (Ai et al., 1999). Moreover, for the cannabinoid 1 receptor, which, like the μ -opioid receptor, couples to a pertussis toxin-sensitive G-protein, reductions in cAMP are required for ERK/MAPK activation in vitro (Davis, Ronesi, & Lovinger, 2003). If extinction requires vPAG ERK/MAPK activation, then extinction should be prevented by inhibition of the ERK/MAPK pathway. The present experiment studies this possibility. Thirty-two rats were first trained to fear an auditory CS via pairing with footshock. The fear that had accrued to that CS was then extinguished. For the control group, Group 0 μg ($n = 8$), extinction was preceded by microinjection of a DMSO vehicle into the vPAG. For the experimental groups, extinction was preceded by infusions of 0.1 μg (Group 0.1 μg ; $n = 8$), 1 μg (Group 1 μg ; $n = 8$), or 5 μg (Group 5 μg ; $n = 8$) of U0126. U0126 is a potent inhibitor of

the kinase activity of MAPKK/MEK, and the doses chosen span a wide dose range, including those that have previously been found to impair amygdala long-term potentiation and acquisition of fear conditioning (Schafe et al., 2000). After 2 days of extinction training, rats were tested, in the absence of U0126, for fear reactions to the auditory CS.

Results and Discussion

Histology. The top panel of Figure 5 shows the location of microinjection tips for rats in this experiment. Seven animals were excluded from the experiment because of incorrect cannula placement. Thus, 25 animals were included in the analyses (Group Vehicle, $n = 8$; Group 0.1 μg , $n = 6$; Group 1 μg , $n = 5$; Group 5 μg , $n = 6$).

Behavior. The results from extinction and test are shown in the middle and bottom panels of Figure 5. The format for this figure is the same as described for Figure 1. There were no differences among groups in pre-CS levels of freezing either on Day 1, $F(1, 21) < 1$, $p > .05$, or at test, $F(1, 21) < 1$, $p > .05$. There is evidence for extinction, because freezing decreased significantly from Day 1 of extinction training to Day 2 of extinction training, $F(1, 21) = 125.1$, $p < .05$. This extinction was unaffected by vPAG infusions of U0126 across a wide dose range. There were no overall differences among groups in freezing during extinction as a linear function of U0126 dose, $F(1, 21) < 1$, $p > .05$. There was also no interaction between the decrease in freezing from Day 1 to Day 2 and U0126 dose, $F(1, 21) < 1$, $p > .05$. Finally, there were no differences among groups on test as a linear function of U0126 dose, $F(1, 21) < 1$, $p > .05$. In other words, there is no evidence from this experiment that activation of vPAG ERK/MAPK contributes to extinction, because infusions of the MEK/MAPKK inhibitor U0126 did not impair the development of fear extinction.

General Discussion

Opioid receptors in the mammalian central nervous system contribute to fear extinction and other associative-based reductions in fear (McNally et al., 2004a; McNally & Westbrook, 2003). This contribution is mediated by opioid receptors in the vPAG (McNally et al., 2004b). The present experiments study the vPAG opioid receptor subtype and signal transduction events that mediate fear extinction. The results identify the μ -opioid receptor as an important opioid receptor subtype for fear extinction. The development of extinction was significantly impaired by vPAG infusions of the selective μ -opioid receptor antagonist CTAP. Moreover, CTAP was effective only when it was infused into the vPAG. Infusions of CTAP dorsal or lateral to the vPAG were significantly less effective. The effects of CTAP on fear extinction in the present experiments were similar to the effects of systemic (McNally & Westbrook, 2003) or intra-vPAG (McNally et al., 2004b) administrations in our previous work. Our previous results showed that concentrations of naloxone as low as 1.2 nM infused into the vPAG impaired fear extinction. At such doses, naloxone has greatest affinity for the μ -receptor (Mansour, Hoversten, Taylor, Watson, & Akil, 1995). An apparent discrepancy between the effects of CTAP reported here and the effects of the nonselective antagonist naloxone on fear extinction reported by us previously is

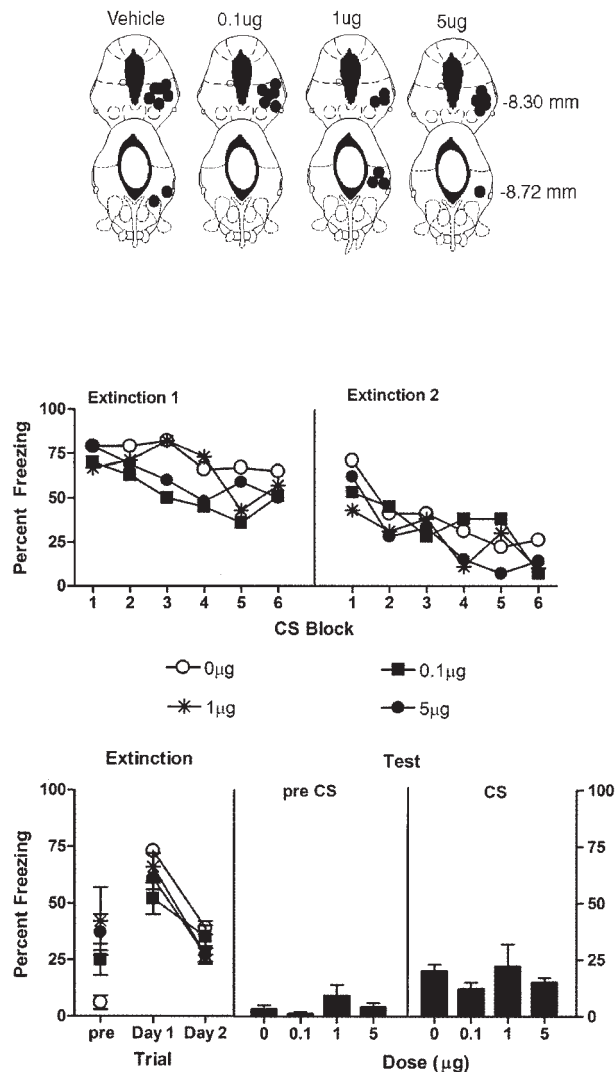


Figure 5. Role of ventrolateral quadrant of the midbrain periaqueductal gray (vlPAG) extracellular signal-regulated kinase/mitogen-activated protein kinase in extinction of conditioned freezing. Top panel: Location of injection cannula placements in the vlPAG. (distances in millimeters from bregma). Reprinted from *The Rat Brain in Stereotaxic Coordinates*, 4th ed., G. Paxinos and C. Watson, Copyright 1998, with permission from Elsevier. Middle panel: Mean ($\pm$ SEM) percentage of freezing per block of two conditioned stimuli (CSs) across the 2 days of extinction training for control rats (Group 0 μ g) microinjected with a dimethyl sulfoxide vehicle and rats microinjected with 0.1 μ g, 1 μ g, or 5 μ g of the MAP kinase kinase (MAPKK/MEK) inhibitor U0126. Bottom panel, mean ($\pm$ SEM) percentage of freezing during the first 2 min prior to CS onset on Day 1 and average freezing across the 2 days of extinction training as well as freezing during the drug-free test for the two groups described above.

the evidence for within-session extinction on Day 2 among the CTAP-treated animals (see Figure 2). By contrast, in our previous work with naloxone and in the present experiment with the cAMP analog 8-Br-cAMP (see Figure 3), there is little evidence for such within-session extinction. This pattern of findings might be interpreted to mean that CTAP impaired consolidation of the extinction memory rather than the learning that underpins extinction. How-

ever, it is worth noting that we have previously directly tested the possibility that opioid receptors regulate consolidation of the extinction memory. We could find no evidence to support this possibility (McNally & Westbrook, 2003). The apparent differences in time course of responding during extinction could be due to the fact CTAP is a peptide and significantly less stable in vivo than either naloxone or 8-Br-cAMP.

It remains possible that the absence of significant evidence for involvement of δ - and κ -opioid receptors was due to a failure to test with an adequate dose of antagonist. However, the importance of vlPAG μ -opioid receptors in fear extinction is supported by the known properties of vlPAG opioid receptors. Electrophysiological evidence identifies the μ -opioid receptor as making the largest contributions to vlPAG opioid actions at the cellular level (Chieng & Christie, 1994). There is little evidence for reliable synaptic effects of δ -opioid receptors in the vlPAG of naive rats. Indeed, in the naive animal, PAG δ -opioid receptors are predominantly localized to cytoplasmic compartments, not the cell surface (Commons, 2003), and do not produce synaptic effects in the naive animal (e.g., Chieng & Christie, 1994; Connor & Christie, 1998; Vaughan & Christie, 1997). Moreover, the effects of κ -opioid receptor manipulations on fear conditioning are typically opposite to the effects of μ -opioid receptor manipulations (e.g., Fanselow et al., 1991). The present results are therefore consistent with known neuroanatomical, electrophysiological, and functional properties of midbrain opioid receptors.

The present results also identify reductions in vlPAG cAMP but not PKA as important for fear extinction. Extinction was impaired, in a dose-dependent manner, by infusions of the membrane-permeable cAMP analog 8-Br-cAMP but was unaffected by the selective PKA activator Sp-cAMPS across a wide dose range, including doses previously shown to prevent learning in other brain regions. This finding is consistent with the well-documented inhibition of adenylyl cyclase and intracellular cAMP by μ -opioid receptor activation (see Williams et al., 2001, for a review). This inhibition contributes to inhibition of the voltage-dependent cation channel I_h (e.g., Ingram & Williams, 1994) and inhibition of neurotransmitter release (see Williams et al., 2001, for a review). However, in the PAG, the role of reduced cAMP in μ -opioid receptor signaling remains unclear. For opioid presynaptic inhibition of gamma-aminobutyric acid (GABA), the most well-studied opioid effect in vlPAG and the primary mechanism for opioid analgesia (Fields, 2004), inhibition of adenylyl cyclase and cAMP is largely unimportant (Vaughan, Ingram, Connor, & Christie, 1997). Rather, inhibition of GABA depends on coupling of vlPAG μ -opioid receptors to a dendrotoxin- and 4-AP-sensitive K^+ channel via phospholipase A_2 , arachidonic acid, and 12-lipoxygenase (Vaughan et al., 1997). Opioid inhibition of GABA can depend on reductions in cAMP and PKA with prolonged opiate exposure (e.g., Ingram, Vaughan, Bagley, Connor, & Christie, 1998), but such mechanisms are unlikely to contribute to fear extinction, because the PKA activator Sp-cAMPS failed to affect development of fear extinction. vlPAG opioid receptors, therefore, do not appear to contribute to fear extinction by the same presynaptic inhibition of GABA that is important for opioid analgesia. The precise cellular consequences of reductions in vlPAG cAMP remain to be elucidated. Regardless, an important implication of our claim that extinction required reductions in vlPAG cAMP is that

extinction should be facilitated by manipulations that reduce vIPAG cAMP.

There is no evidence from these experiments that alteration in vIPAG PKA or ERK/MAPK signaling contributes to fear extinction. The failures of the PKA activator and the ERK/MAPK inhibitor to modulate fear extinction are interesting because extinction does not simply involve erasure of the CS–shock association. Rather, several lines of evidence indicate that the original CS–shock association remains largely intact after extinction training (for reviews, see Delamater, 2004; Rescorla, 2001). Fear extinction is therefore believed to involve new learning, although the contents of this learning remain unclear (for reviews, see Delamater, 2004; Rescorla, 2004). Because extinction is held to involve new learning, it has been hypothesized that extinction should be mediated by the same structures, neurotransmitters, and cell-signaling cascades as other forms of learning (Davis, 2002; Myers & Davis, 2002). Indeed, in the amygdala and hippocampus, two structures important for fear learning, acquisition and extinction of fear are both dependent on activation of NMDA receptors, and they share intracellular signaling mechanisms, including activation of cAMP, PKA, and MAPK (Lin, Yeh, & Gean, 2003; Lu et al., 2001; Szapiro et al., 2003), that have been implicated in multiple forms of synaptic plasticity and learning (e.g., Bailey, Bartsch, & Kandel, 1996; Kandel, 2001; Sharma & Carew, 2004). In the vIPAG, activation of any of these signaling pathways does not contribute to fear extinction. In fact, the present experiments show the opposite. In the vIPAG, fear extinction requires reductions in cAMP because extinction can be prevented, and fear maintained, in a dose-dependent manner by a cAMP analog.

Our findings are counterintuitive from the perspective that extinction is a new learning process and that such processes typically involve increased cAMP–PKA and/or ERK/MAPK signaling. However, the previously published findings from the amygdala and hippocampus showing that fear acquisition and fear extinction share intracellular mechanisms (e.g., cAMP–PKA, ERK/MAPK) are counterintuitive from the perspective that the behavioral circumstances producing fear acquisition versus fear extinction are different. According to error correction–based accounts of the conditioning process, fear acquisition occurs when the actual outcome of the conditioning trial exceeds the expected outcome, whereas fear extinction occurs when the expected outcome exceeds the actual outcome. On the basis of the ability of opioid receptor antagonists to facilitate the acquisition of fear (e.g., McNally et al., 2004a; Young & Fanselow, 1992) but prevent its extinction (McNally et al., 2004b; McNally & Westbrook, 2003), overexpectation (McNally et al., 2004a), and associative blocking (McNally et al., 2004a), we have suggested that opioid receptors contribute to the error correction process during conditioning (McNally et al., 2004a, 2004b). Specifically, our suggestion is that during fear conditioning, opioid receptors encode the $-V$ term in the delta rule ($\lambda - \Sigma V$; Rescorla & Wagner, 1972). Opioid receptor antagonists impair extinction because they reduce the discrepancy between the expected (V) and actual (λ) outcomes by reducing the value of $-V$ in ($\lambda - \Sigma V$), where $\lambda = 0$. So, during nonreinforcement (e.g., extinction) or whenever the expected outcome (V) exceeds the actual outcome (λ ; e.g., overexpectation), activation of opioid receptors provides the negative discrepancy or error signal that initiates synaptic plasticity and extinction association formation elsewhere in the brain. The present results showing

that fear extinction requires reductions in vIPAG cAMP are consistent with this symmetry between the conditions promoting fear acquisition versus fear extinction.

Extinction-like treatments (e.g., exposure, systematic desensitization) remain among the most effective interventions for anxiety disorders in humans. Recent work has shown that administrations of an NMDA receptor partial agonist, which facilitates fear extinction in rats (Walker et al., 2002), also enhances the efficacy of exposure therapy for simple phobia (Ressler et al., 2004). Activation of opioid receptors contributes to the efficacy of behavioral interventions for simple phobia. Administrations of opioid receptor antagonists (e.g., naltrexone or naloxone) prior to exposure therapy or systematic desensitization for simple phobia significantly reduce the therapeutic benefit of those treatments (Arntz, Merkelbach, & de Jong, 1993; Egan, Carr, Hunt, & Adamson, 1988; Merluzzi, Taylor, Boltwood, & Götestam, 1991). Given the important role of the endogenous opioid system in fear extinction and behavioral treatments of anxiety disorders, it is possible that manipulations that enhance the activity of the endogenous opioid system enhance the efficacy of these treatments. Our identification of the opioid receptor subtype and signal transduction mechanism for vIPAG contributions to fear extinction might therefore help identify novel targets and adjuncts for the treatment of anxiety disorders. Current research in our laboratory is addressing this issue.

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Received January 7, 2005

Revision received March 16, 2005

Accepted April 21, 2005 ■

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