Research

The effect of the μ -opioid receptor antagonist naloxone on extinction of conditioned fear in the developing rat

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Several recent studies report that neurotransmitters that are critically involved in extinction in adult rats are not important for extinction in young rats. Specifically, pretest injection of the γ -aminobutyric acid (GABA) receptor inverse agonist FG7142 has no effect on extinction in postnatal day (P)17 rats, although it reverses extinction in P24 rats as reported by Kim and Richardson in an earlier paper. Further, pre-extinction injection of the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 has no effect on extinction in P17 rats, whereas it impairs long-term extinction in P24 rats as per Langton and colleagues in an earlier work. These findings indicate that extinction in P17 rats is qualitatively different from extinction in older rats. The present study examines the involvement of the endogenous opioid system in extinction in the developing rat using systemic injections of the μ -opioid receptor antagonist naloxone. Experiment 1 showed that injection of naloxone before extinction training disrupted the acquisition of extinction in both P17 and P24 rats. This effect was dependent on central rather than peripheral μ -opioid receptors (Experiment 2), and neither pre-test nor post-extinction injection of naloxone had effects on extinction (Experiments 3 and 4). Taken together, these findings indicate that opioid neurotransmission, in contrast to GABA and NMDA activity, is critical for extinction acquisition across development.

Pavlovian conditioned fear is typically acquired by pairing an initially neutral conditioned stimulus (CS; e.g., tone) with an aversive unconditioned stimulus (US; e.g., shock). The formation of an association between the CS and the US is inferred when subsequent presentations of the CS elicits various conditioned fear responses (CR; e.g., freezing). These learned fear responses can subsequently be reduced by giving nonreinforced presentations of the CS—a process referred to as extinction. Fear extinction has received considerable attention over the past decade because of its theoretical importance and its obvious clinical implications for the treatment of various anxiety disorders (Davis and Myers 2002). Early models of associative learning suggested that extinction was due to the “unlearning” or “erasure” of the original CS–US association (e.g., Rescorla and Wagner 1972). However, it is now more widely accepted that the reduced CR following extinction reflects new learning of a CS–no US association that inhibits the expression of the original CS–US association (e.g., Bouton 2002). The primary evidence for this view comes from behavioral studies that show performance to an extinguished CS can recover without any retraining (e.g., spontaneous recovery, renewal, and reinstatement). Additionally, we know that extinction involves inhibition because reducing γ -amino butyric acid (GABA) inhibitory activity by pretest injections of the GABA receptor inverse agonist FG7142 reverses extinction (Harris and Westbrook 1998). Finally, pre-extinction injection of the *N*-methyl-D-aspartate (NMDA) antagonist MK-801 impairs long-term extinction (Baker and Azorlosa 1996); the critical role of NMDA in new learning is well known (e.g., Miserendino et al. 1990; Kandel 2001).

However, several recent studies suggest that extinction that occurs early in development is not new inhibitory learning. These studies report that postnatal day (P)17 rats fail to show renewal or

reinstatement of extinguished fear, whereas P24 rats do (all age labels refer to the age at extinction training) (Kim and Richardson 2007a,b; Yap and Richardson 2007). Also, pretest injection of FG7142 has no effect on extinction in P17 rats, although it reverses extinction in P24 rats (Kim and Richardson 2007a). Further, pre-extinction injection of MK-801 has no effect on long-term extinction in P17 rats, whereas long-term extinction in P24 rats is impaired (Langton et al. 2007). These findings indicate that extinction in P17 rats is not new inhibitory learning, and that extinction at this age is qualitatively different from extinction in P24 and adult rats. Not only are these findings important theoretically, the observed developmental differences in extinction also have significant clinical implications. In contrast to suggestions that fear acquired early in development has enduring effects that are resistant to extinction (Jacobs and Nadel 1985, 1999), these preclinical studies suggest that fear acquired early in development may be more permanently extinguished if treated early compared with later in development.

The present study further investigates extinction in the developing rat by examining the involvement of the endogenous opioid system. Along with GABAergic and NMDA neurotransmission, endogenous opioids regulate extinction in the adult rat (Hernandez and Powell 1980, 1983; McNally and Westbrook 2003a; McNally et al. 2004, 2005). For example, pre-extinction injection of the μ -opioid receptor antagonist naloxone significantly impaired the acquisition of extinction (McNally and Westbrook 2003a). That is, rats given naloxone before extinction exhibited significant CS-elicited freezing across the CS-alone presentations and did not exhibit substantial within-session extinction learning, in contrast to vehicle-treated rats. Also, neither post-extinction nor pretest injection of naloxone had any effects, providing evidence that opioid neurotransmission is critical for the acquisition of extinction rather than its consolidation or expression. This finding is interesting because the importance of NMDA and GABA in extinction is mainly on the consolidation of extinction learning

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or the expression of extinction memory, respectively. Therefore, it may be the case that reducing NMDA and GABA activity in P17 rats has no effects on extinction because extinction at this age does not involve the consolidation or the expression of an extinction memory (e.g., extinction at this age may be erasure). However, pre-extinction injection of naloxone may disrupt extinction in P17 rats, because opioid neurotransmission appears to be important specifically for the acquisition of extinction. Therefore, in Experiment 1 we gave a systemic injection of naloxone before extinction in P17 and P24 rats. We expected naloxone to impair extinction acquisition in P24 rats because previous findings showed that P24 rats essentially are like adult rats in extinction. Whether naloxone attenuates extinction in P17 rats was our primary question of interest.

Results

Acquisition of extinction in P17 and P24 rats is opioid dependent

Pre-extinction injection of the μ -opioid receptor antagonist naloxone has been shown to significantly impair extinction acquisition in the adult rat (McNally and Westbrook 2003a). To examine opioid involvement in extinction in the developing rat, we gave P17 and P24 rats a systemic injection of saline or naloxone (5 mg/kg) 10 min before extinction training.

Pre-extinction injection of naloxone significantly impaired the acquisition of extinction in both P17 and P24 rats (Fig. 1A). A mixed-design analysis of variance (ANOVA) of the extinction data yielded significant main effects of Block ($F_{(3,99)} = 10.1$, $P < 0.005$) and Drug ($F_{(1,33)} = 6.0$, $P < 0.05$), as well as a Block $\times$ Drug interaction ($F_{(3,99)} = 15.5$, $P < 0.0001$). There were no other significant main effects or interactions (biggest $F = 3.2$). The mean levels of CS-elicited freezing at test are shown in Figure 1B. ANOVA did not reveal any significant group differences in baseline freezing levels at test in any experiment (Table 1 displays the baseline freezing levels for each group in all experiments). Rats that received a pre-extinction injection of naloxone exhibited substantially higher levels of CS-elicited freezing at test (drug-free) compared with rats that had received saline. There was a main effect of Drug ($F_{(1,33)} = 9.5$, $P < 0.005$), but there was no effect of Age or Drug $\times$ Age interaction ($F_s < 1$). These results show that μ -opioid neurotransmission is involved in extinction acquisition in P17 and P24 rats.

Figure 1. The mean ($\pm$ SEM) levels of CS-elicited freezing during extinction and test in Experiment 1. (A) Pre-extinction injection of naloxone impaired the acquisition of extinction in P17 and P24 rats. (B) Groups P17-Naloxone ($n = 9$) and P24-Naloxone ($n = 10$) displayed significantly impaired extinction than groups P17-Saline ($n = 9$) and P24-Saline ($n = 9$) at test.

Table 1. Mean ($\pm$) SEM % freezing at baseline test

Experiment	Groups	Mean % freezing at baseline
1	P17-Saline	15 ($\pm$ 5)
	P17-Naloxone	8 ($\pm$ 4)
	P24-Saline	9 ($\pm$ 3)
	P24-Naloxone	10 ($\pm$ 2)
2	Saline	7 ($\pm$ 3)
	Naloxone	7 ($\pm$ 2)
	Naloxone Methiodide	3 ($\pm$ 1)
3	Saline-Saline	7 ($\pm$ 3)
	Saline-Naloxone	13 ($\pm$ 3)
	Naloxone-Saline	5 ($\pm$ 3)
	Naloxone-Naloxone	10 ($\pm$ 3)
4	No extinction	4 ($\pm$ 2)
	Pre-extinction Naloxone	6 ($\pm$ 4)
	Post-extinction Saline	9 ($\pm$ 4)
	Post-extinction Naloxone	6 ($\pm$ 3)

There were no significant group differences in any experiment.

Central, not peripheral, μ -opioid receptors are critical for the acquisition of extinction in P17 rats

It was demonstrated in Experiment 1 that pre-extinction systemic injection of naloxone significantly impairs extinction learning in P17 and P24 rats. The result with the P24 rat is consistent with previous findings with adult rats and further shows that P24 rats are adult-like in terms of extinction. On the other hand, finding that naloxone affects extinction acquisition in P17 rat is quite novel considering that no previous pharmacological agent has been shown to affect extinction at this age (i.e., neither MK-801 nor FG7142 affect extinction at this age). Therefore, follow-up experiments in this study focused on extinction in P17 rats. Experiment 2 examined whether the effect of naloxone on extinction in rats this age is centrally or peripherally mediated by using naloxone methiodide (which does not readily cross the blood-brain barrier). Ten minutes before extinction, P17 rats were injected with saline, naloxone, or naloxone methiodide.

Pre-extinction injection of naloxone significantly impaired the acquisition of extinction, whereas saline and naloxone methiodide had no effects (Fig. 2A). A mixed-design ANOVA revealed significant main effects of Block ($F_{(3,81)} = 10.2$, $P < 0.005$) and Drug ($F_{(2,27)} = 5.3$, $P < 0.05$); the Block $\times$ Drug interaction approached significance ($F_{(3,81)} = 5.3$, $P = 0.059$). The mean levels of CS-elicited freezing at test are shown in Figure 2B. Experiment 2 replicated Experiment 1 in that P17 rats that received a pre-extinction injection of naloxone showed significantly higher levels of CS-elicited freezing (i.e., impaired extinction) compared with rats that had received saline. Injection of naloxone methiodide, however, had no effects on extinction in P17 rats; that is, these rats responded at test as did rats given saline. An ANOVA of CS-elicited freezing levels at test showed a significant effect of Drug ($F_{(2,27)} = 4.3$, $P < 0.05$). Post-hoc student-Newman-Keuls (SNK) pairwise comparisons showed that group naloxone exhibited significantly higher levels of freezing than did either of the other two groups ($P_s < 0.05$). This result indicates that the effect of naloxone on extinction in the P17 rat is mediated by central opioid receptors, as has been reported in adult rats (McNally and Westbrook 2003a).

The effect of naloxone on extinction is not due to state dependency

Experiments 1 and 2 showed that pre-extinction injection of naloxone significantly impairs extinction acquisition in P17 rats. Additionally, this effect was shown to be mediated by central, not peripheral, μ -opioid receptors. However, it may be the case that pre-extinction injection of naloxone merely causes freezing in P17

Figure 2. The mean ($\pm$ SEM) levels of CS-elicited freezing during extinction and test in Experiment 2. (A) Pre-extinction injection of naloxone, but not naloxone methiodide, impaired the acquisition of extinction in P17 rats. (B) Group Naloxone ($n = 11$) displayed significantly impaired extinction than groups Saline ($n = 9$) and Naloxone Methiodide ($n = 10$) at test.

rats. Further, rats injected with naloxone before extinction may not be able to express any extinction learning at test because of the change in internal context. Therefore, the next experiment was designed to assess both of these possibilities by giving P17 rats either saline or naloxone before extinction as well as before test.

Consistent with the previous experiments, pre-extinction injection of naloxone significantly impaired the acquisition of extinction compared with pre-extinction injection of saline (Fig. 3A). A mixed-design ANOVA revealed significant main effects of Block ($F_{(3,138)} = 15.0$, $P < 0.0001$) and Pre-extinction drug ($F_{(1,46)} = 6.8$, $P < 0.05$), as well as a significant Block $\times$ Pre-extinction drug interaction ($F_{(3,138)} = 4.9$, $P < 0.05$). There was no effect of Pre-test drug, or any other significant interactions ($F_s < 1$). The mean levels of CS-elicited freezing at test are shown in Figure 3B. Pre-extinction administration of naloxone impaired the acquisition of extinction regardless of the drug given before test. Analysis of CS-elicited freezing at test yielded a main effect of Pre-extinction drug ($F_{(1,46)} = 9.8$, $P < 0.005$), but there was no effect of Pre-test drug or an interaction of the two factors ($F_s < 1$). These results indicate that injection of naloxone does not merely increase freezing, because rats that received saline before extinction and naloxone before test exhibited low levels of freezing (i.e., these rats expressed extinction at test). Also, the finding that naloxone does not affect freezing during test in these rats shows that the persistent freezing during extinction following injection of naloxone is not a performance effect; rather, naloxone appears to cause a deficit in extinction acquisition. Further, it appears that the effect of naloxone on extinction acquisition is not a state-dependent effect, because giving these rats a pre-test injection of naloxone did not lead to the expression of extinction.

Opioid neurotransmission is not involved in the consolidation of extinction in P17 rats

A previous study showed that opioid neurotransmission is critical for the acquisition of extinction rather than its consolidation, because post-extinction injection of naloxone had no effects in the adult rat (McNally and Westbrook 2003a). Therefore, Experiment 4 assessed whether naloxone affects the consolidation of extinction in P17 rats. Three groups of rats were either given naloxone or saline immediately after extinction, or given naloxone before extinction. Another group of rats was initially trained, but then did not receive any CS-alone trials; these rats were only

exposed to the context for an equivalent period of time as the extinguished rats.

During extinction training, rats that were given naloxone before extinction expressed impaired extinction acquisition (Fig. 4A). The groups that received post-extinction injections of either saline or naloxone expressed high CS-elicited levels of freezing in the first block of extinction that decreased significantly by the end of extinction. A mixed-design ANOVA of these data revealed significant main effects of Block ($F_{(3,54)} = 15.0$, $P < 0.0001$) and Group ($F_{(2,18)} = 8.8$, $P < 0.005$), as well as a significant Block $\times$ Group interaction ($F_{(6,54)} = 5.3$, $P < 0.05$). The mean levels of CS-elicited freezing at test are shown in Figure 4B. Rats given a post-extinction injection of naloxone performed at test similarly to rats given saline, but rats given a pre-extinction injection of naloxone exhibited impaired extinction at test. Rats that did not receive extinction training showed a high level of CS-elicited freezing, as expected. An overall ANOVA of these data showed a significant effect of Group ($F_{(3,25)} = 10.1$, $P < 0.0001$). Post-hoc SNK pairwise comparisons showed that groups No extinction and Pre-extinction naloxone exhibited significantly higher levels of freezing at test than did the other two groups ($P_s < 0.05$). These results show that the effect of naloxone is on the acquisition of extinction rather than its consolidation.

Discussion

Several recent studies have reported that P17 rats fail to exhibit a variety of extinction-related phenomena, whereas P24 rats do (Kim and Richardson 2007a,b; Langton et al. 2007; Yap and Richardson 2007). To extend these findings, we examined the effect of the μ -opioid receptor antagonist naloxone on extinction in the developing rat. We showed that pre-extinction injection of naloxone significantly impaired the acquisition of extinction in both P17 and P24 rats (Experiment 1). This effect was mediated by central, not peripheral, opioid receptors (Experiment 2). Additionally, naloxone injection immediately after extinction or before test had no effects on extinction in P17 rats (Experiments 3 and 4). These results indicate that endogenous opioid neurotransmission is critically important for the acquisition of extinction across development.

Extinction of conditioned fear in the developing rat

In adult rats, it is widely accepted that multiple neurotransmitter systems are involved in extinction of conditioned fear (for review, see Myers and Davis 2007). The major ones include the NMDA, GABA, and endogenous μ -opioid system. It may be the case that

Figure 3. The mean ($\pm$ SEM) levels of CS-elicited freezing during extinction and test in Experiment 3. (A) Pre-extinction injection of naloxone impaired the acquisition of extinction in P17 rats. (B) Groups Naloxone–Saline ($n = 12$) and Naloxone–Naloxone ($n = 12$) displayed significantly impaired extinction than groups Saline–Saline ($n = 14$) and Saline–Naloxone ($n = 12$) at test.

Figure 4. The mean ($\pm$ SEM) levels of CS-elicited freezing during extinction and test in Experiment 4. (A) Pre-extinction injection of naloxone impaired the acquisition of extinction in P17 rats. (B) Groups No Extinction ($n = 8$) and Pre-extinction Naloxone ($n = 7$) displayed significantly higher levels of freezing compared with groups Post-extinction Saline ($n = 7$) and Post-extinction Naloxone ($n = 7$) at test.

having multiple neurotransmitters involved in extinction allows for different mechanisms to mediate extinction in the adult rat. Numerous recent reviews on extinction now suggest both “new learning” and “erasure” as mechanisms for extinction (Delamater 2004; Barad et al. 2006; Lattal et al. 2006; Myers and Davis 2007). One of the reasons for this reintroduction of “unlearning” as a potential mechanism for extinction is that recovery of extinguished fear (e.g., renewal, spontaneous recovery) is seldom complete (for review, see Delamater 2004). Another reason is that extinction leads to permanent reductions in synaptic potentials in some amygdala neurons that expressed increased synaptic potentials as a result of previous conditioning (for review, see Barad et al. 2006). Therefore, the dominant mechanism for extinction may be new learning, but that does not rule out erasure as a possible mechanism mediating extinction in the adult rat (Lattal and Stafford 2008).

The balance between erasure and new learning may be simply shifted when extinction occurs early in development compared with when it occurs in adulthood, such that extinction relies more on erasure than new learning. As mentioned earlier, P17 rats fail to show renewal or reinstatement of extinguished fear (Kim and Richardson 2007a,b; Yap and Richardson 2007). Also, pre-test injection of FG7142 has no effect on extinction in P17 rats although it reverses extinction in P24 rats (Kim and Richardson 2007a). These findings strongly suggest that extinction in P17 rats is erasure. Particularly striking is the recent finding that the amygdala is critical for re-extinction in P17 rats (Kim and Richardson 2008). In the P24 and adult rat, the amygdala is particularly important only for extinction the first time, but appears unimportant for re-extinction (Kim and Richardson 2008; Laurent et al. 2008). That is, inactivation of the amygdala prior to the first extinction session markedly impairs extinction acquisition (i.e., these rats express high levels of freezing at a subsequent test). In contrast, when a fear-eliciting CS was extinguished, reconditioned, and then re-extinguished, P24 and adult rats exhibited low levels of freezing (i.e., good extinction) the next day regardless of whether the amygdala was inactivated or not at the time of re-extinction. From this it appears that the amygdala is involved in the initial learning of the CS–no US memory acquired in extinction training, but once that memory has been acquired, then the amygdala is no longer needed for subsequent extinction training episodes, at least in the adult rat. However, Kim and Richardson (2008) showed that re-extinction is amygdala dependent if initial extinction occurs at 17 d of age and amygdala independent if initial extinction occurred at 24 d of age, even when training,

retraining, re-extinction, and test age was kept constant. This finding strongly suggests that extinction involves unlearning of the original CS–US association in P17 rats; thus, subsequent re-extinction still requires the amygdala.

We propose that extinction may be erasure in P17 rats, because extinction at this age relies heavily on the opioid system rather than the multiple neurotransmitter systems that are involved in extinction in the adult rat. The endogenous opioid system may be involved in erasure of conditioned fear during extinction in P17 rats because there is evidence showing that the activation of opioid system disrupts consolidation of memory in adult rats. For example, postconditioning injections of opioid receptor agonists disrupt long-term memory (e.g., Rudy et al. 1999; McNally and Westbrook 2003b). Therefore, increased opioid neurotransmission triggered by extinction training may cause some unlearning of the fear memory. The endogenous opioids also play an important role in experimentally induced amnesia. Specifically, Rudy and his colleagues proposed that amnesia due to post-conditioning social isolation is a failure in consolidation of memory due to opioid release, because opioid receptor antagonists prevent this form of amnesia (Rudy et al. 1999). From this, it could be said that opioid neurotransmission can “erase” memory because it prevents and/or reverses consolidation. In fact, the endogenous opioid system is active from birth (Blass et al. 1991), and has been found to modulate memory in infant rats (Weber et al. 2006). Furthermore, the endogenous opioid system is particularly active early in development, because behaviors specific to infancy, such as suckling and play, have been found to activate the opioid system. For example, intraoral infusions of milk in 10-d-old rats reduce distress vocalizations and increase analgesia to aversive stimulation, effects that are prevented by opioid receptor antagonists (Blass and Fitzgerald 1988). Play behavior appears to also activate the opioid system—opioid receptor antagonists reduce social play and opioid agonists increase it (Vanderschuren et al. 1995, 1996). From these studies, endogenous opioid activity would likely be higher in infant rats compared with older rats. Considering the effectiveness of opioids in inducing post-conditioning amnesia, the involvement of a particularly active endogenous opioid system in extinction at P17 may lead to erasure of the original conditioned fear.

Opioid neurotransmission in fear extinction

The opioid-based erasure hypothesis is consistent with recent evidence on the role of μ -opioids in associative learning. Some theories of associative learning posit that in order for learning to occur, animals need to detect an error between the actual and the expected outcome. These theories also predict that the error detection occurring during extinction leads to unlearning of the original CS–US association (Rescorla and Wagner 1972). Interestingly, McNally and his colleagues (McNally et al. 2004, 2005; McNally and Cole 2006) provided evidence showing that opioid neurotransmission in the ventrolateral periaqueductal gray (vlPAG) is critically important for error detection. Based on this, if extinction in P17 rats is due to unlearning, then it would be opioid dependent, which is consistent with the present findings.

It is presently unknown whether extinction in P17 rats is also dependent on μ -opioid receptors in the vlPAG, and future studies should explore this issue. If that is the case, then it would provide support for the idea that unlearning may be a shared mechanism for extinction in P17 and adult rats. As mentioned previously, although the dominant mechanism for extinction in the adult appears to be new learning, there is increasing appreciation for the notion that unlearning may also be involved in extinction in the adult rat (Delamater 2004; Barad et al. 2006; Lattal et al. 2006; Myers and Davis 2007; Lattal and Stafford 2008). If it is the case that opioid neurotransmission has a similar role in extinction

across development, then the partial impairment in extinction produced by naloxone in adult rats, in contrast to the almost complete impairment in extinction observed in P17 rats in the present study, would be a reflection of the differential contribution of the unlearning process to extinction across development. That is, extinction early in development is largely mediated by unlearning, while extinction in older rats consists of some unlearning as well as the learning of a CS–no US association. Overall, opioid neurotransmission may be responsible for the unlearning aspect of fear extinction across development, whereas GABAergic and NMDA neurotransmission underlies the new learning aspect of extinction in the adult rat.

The present findings also predict that increasing opioid neurotransmission should facilitate extinction in the adult rat. Indeed, McNally (2005) recently demonstrated that increasing opioid neurotransmission in the vPAG by a microinfusion of the enkephalin-degrading enzyme inhibitor RB101(S) significantly facilitated extinction to an auditory CS that had been previously paired with shock. In contrast, Morris and Gebhart (1978) reported that pre-extinction systemic injection of morphine led to impaired extinction at test the next day. However, the results reported by Morris and Gebhart (1978) are somewhat difficult to interpret because within-session extinction performance was not measured. Therefore, it may have been the case that the levels of initial learning were different across the groups, or that the extinction training was not sufficient to cause any decrease in performance (i.e., performance was still very high across all groups at test). In McNally (2005), the effect of increasing opioid neurotransmission on extinction was only observed on the second day of extinction training, suggesting that some amount of extinction needs to occur to see any facilitation of extinction. Further studies are necessary to clarify this issue.

It should be noted that other neurotransmitter systems such as the adrenergic and endogenous cannabinoid systems, as well as L-voltage-gated calcium channels (L-VGCC), are also involved in extinction in the adult rat (Myers and Davis 2007). However, the role of adrenergic and endocannabinoid system in extinction appears to depend on GABAergic neurotransmission (e.g., Marsicano et al. 2002; Azad et al. 2003; Berlaui and McGaugh 2006). Therefore, those neurotransmitter systems may not be important for extinction in P17 rats, because it has been demonstrated that alterations in GABAergic activity does not affect an extinction at 17 d of age (Kim and Richardson 2007a). Nevertheless, the potential importance of adrenergic, endocannabinoid, L-VGCC, and other neurotransmitter systems in extinction in P17 rats cannot be determined without additional research.

It is clear that more work needs to be done in order to examine extinction during development. Although extinction is procedurally simple, it is not completely understood at either the theoretical or neural level, and assessing extinction in the developing rat provides a unique way of exploring both of these levels of analysis. Also, understanding extinction early in development is important because of the long-held belief that early learning experiences have a profound impact on later behavior (Mineka and Zinbarg 2006) and longitudinal data suggest that anxiety disorders are the most prevalent disorders in childhood (Newman et al. 1996). The present findings, along with other recent evidence, suggest that although fear is readily acquired early in development, fear is also easily treated early in development.

Materials and Methods

Subjects

Experimentally naïve Sprague-Dawley derived rats were bred and housed in the School of Psychology, University of New South

Wales. All rats were male, and no more than one rat per litter was used per group. Rats were housed with their littermates and mother in plastic boxes (24.5 cm long × 37 cm wide × 27 cm high) covered by a wire lid. Animals were maintained on a 12-h light/dark cycle (lights on at 6 AM) with food and water available *ad libitum*. All animals were treated according to the principles of animal use outlined in The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition), and all procedures were approved by the Animal Care and Ethics Committee at the University of New South Wales.

Drug

Naloxone hydrochloride (N7758, Sigma-Aldrich) and naloxone methiodide (N129, Sigma-Aldrich) were dissolved in 0.9% (weight/volume) sterile physiological saline. Rats were injected subcutaneously with either saline, 5 mg/kg naloxone, or 5.87 mg/kg (this dose was equimolar to the dose of naloxone) naloxone methiodide. Naloxone, naloxone methiodide, and saline were injected in a volume of 2 mL/kg.

Apparatus

Two types of experimental chambers were used to provide different contexts. One type was rectangular (13.5 cm long × 9 cm wide × 9 cm high), with the front wall, rear wall, and ceiling constructed of clear Plexiglas. The floor and side walls consisted of 3-mm stainless steel rods set 1 cm apart. Two high-frequency speakers were located 8 cm from either side of the chamber. A custom-built constant-current shock generator could deliver electric shock to the floor of the chamber as required. A tray of bedding was placed 10 cm below the grid floor. This chamber was housed within a separate wood cabinet with an infrared light. A low, constant background noise (50 dB) was produced by a ventilation fan located within the cabinet. The second type was rectangular (30 cm long × 30 cm wide × 23 cm high) and wholly constructed of Plexiglas, with the exception of the grid floor, which was the same as in the first set of chambers. A clear Plexiglas sheet (35 cm × 35 cm) was placed beneath the grid floor. All of the walls were transparent, except for two side walls that consisted of vertical black and white stripes (5 cm each). Two high-frequency speakers were mounted on the ceiling of each of these chambers. This chamber was housed within a separate wood cabinet with an infrared and a white light-emitting diode. A low, constant background noise (48 dB) was produced by a ventilation fan located within the cabinet. Thus, these two sets of contexts differed primarily in terms of their size and in their visual features. All training occurred in the first type, and extinction and test occurred in the second type of chamber.

The conditioned stimulus (CS) was a white noise; noise level was increased by 8 dB when the CS was presented. A computer controlled all presentations of the CS and the foot shock US. The software and hardware used were developed at the University of New South Wales.

Procedures

Training

Rats were placed in an experimental chamber, and after a 2-min adaptation period, the CS was presented for 10 sec. The shock US (0.6 mA, 1 sec) was administered during the last second of the CS. P17 rats received six pairings in all experiments, whereas P24 rats received three pairings of the CS and US in Experiment 1. The intertrial interval (ITI) ranged from 85 to 135 sec with a mean of 110 sec. Previous research had shown that these training parameters lead to comparable levels of freezing when rats these ages are tested the following day.

Extinction

The extinction session commenced 24 h after training and consisted of the presentation of four CSs in the absence of shock. After a 2-min adaptation period, the 1-min CS was presented with a 1-min ITI.

Testing

Rats were placed in an experimental chamber and their baseline level of freezing in the absence of the CS was recorded for 1 min. The CS was then presented, and freezing was recorded for 2 min. Freezing was scored by a time-sampling procedure, whereby each rat was scored every 3 sec as freezing or not freezing. Freezing was defined as the absence of all movement other than that required for respiration (Blanchard and Blanchard 1969).

Baseline criterion for CS tests and statistical analyses

CS-elicited freezing is difficult to detect if rats display high baseline levels of freezing. Therefore, a baseline criterion was introduced. Specifically, if a rat was freezing more than 40% of the pre-CS period in test, it was not tested. In Experiment 1, one rat each from groups P17-Saline, P24-Saline, and P24-Naloxone were removed. In Experiment 2, one rat from group Saline was removed. In Experiment 3, one rat each from groups Saline-Naloxone and Naloxone-Saline were removed. In Experiment 4, one rat each from groups Pre-extinction Naloxone and Post-extinction Naloxone were removed.

One rat each from groups Saline and Naloxone Methiodide in Experiment 2 and one rat from group Post-extinction Naloxone in Experiment 4 were excluded from the statistical analysis because they were outliers at test (more than three standard deviations away from the group mean).

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