

Maternal Separation Results in Early Emergence of Adult-Like Fear and Extinction Learning in Infant Rats

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Recent studies in rats have shown that extinction occurring early in life is resistant to relapse and may represent the erasure of fear memories. In the present study we examined the effects of early life stress on extinction in the developing rat, which could have important implications for the treatment of anxiety disorders in those who have experienced early life stress. In the present study, we used maternal separation on postnatal days (P) 2–14 as an early life stressor. On P17, maternally separated and standard-reared animals were trained to fear a noise associated with a footshock. The fear of this noise was then extinguished (through repeated nonreinforced noise presentations) on P18. Animals were tested for contextually mediated, stress-mediated, and GABA-mediated fear relapse the day after extinction. We found that young animals exposed to maternal separation were more likely to exhibit context- and stress-mediated relapse after extinction than standard-reared animals (Experiments 1 and 2). Further, unlike standard-reared animals, maternally separated rats exhibited a return of fear when the inhibitory neurotransmitter GABA was blocked at test (Experiment 3). These effects were not the result of maternal separation increasing rats' sensitivity to footshock (Experiment 5) and may in part be related to superior long-term memory for contexts in P17 maternally separated rats (Experiment 4). Taken together, these results suggest that early life adversity may prepare young animals to respond more cautiously toward fear signals in their environment.

Keywords: maternal-separation, stress, development, extinction, fear conditioning

Fear learning and fear inhibition is modeled in rats using the process of Pavlovian fear conditioning and extinction, respectively. Typically, Pavlovian fear conditioning involves pairing a neutral conditioned stimulus (such as a noise) with an unconditioned stimulus (US; such as a shock). Following such pairings the conditioned stimulus (CS) elicits a number of fear responses (e.g., freezing, potentiated startle, changes in heart rate). However, the fear elicited by this CS can be reduced by repeated exposure to the CS in the absence of the US (extinction). Using these procedures, recent research has shown that fear extinction occurring early in development differs dramatically from fear extinction occurring later in development. Extinction occurring from postnatal day (P) 24 through to adulthood involves GABA-mediated inhibition and is dependent on amygdala and infralimbic prefrontal cortex (ilPFC) activity (Harris & Westbrook, 1998; Kim, Hamlin, & Richardson, 2009; Kim & Richardson, 2007b, 2008; Millad & Quirk, 2002). In addition, animals this age are susceptible to fear relapse after extinction (Kim & Richardson, 2007a, 2007b), suggesting that extinction involves new inhibitory learning that temporarily masks the expression of the CS-US association (Bouton, 2002). In contrast, when extinction takes place early in development (i.e.,

P16–18) ilPFC activity and GABAergic inhibition is not required and fear relapse is not exhibited (Gogolla, Caroni, Lüthi, & Herry, 2009; Kim et al., 2009; Kim & Richardson, 2007a, 2007b; Yap & Richardson, 2007). Hence, it has been suggested that while adult extinction is relapse-prone, infant extinction is relapse-resistant and may involve fear erasure (Gogolla et al., 2009; Kim & Richardson, 2008).

As noted above, the ilPFC is not involved when the early developing extinction system is online (Kim et al., 2009), presumably because of the immaturity of the medial PFC (mPFC) at this young age (VanEden & Uylings, 1985). Hence, it is possible that early life manipulations that impact on the mPFC might affect the developmental shift in the mechanisms underlying extinction learning; for example, if the manipulation enhanced development of the mPFC, then these animals may use the later-maturing extinction system at a younger age, with all of the associated consequences (e.g., expression of relapse). Maternal-separation is a stressor that has been shown to transiently increase brain-derived neurotrophic factor (BDNF) mRNA in the PFC of P17 animals (Roceri et al., 2004). Further, epigenetic regulation and expression of the BDNF gene in the PFC has been shown to be critical for adult extinction learning and retention (Bredy, Sun, & Kobor, 2009; Peters, Dieppa-Perea, Melendez, & Quirk, 2010). In addition, early developmental expression of BDNF accelerates the maturation of intracortical GABAergic innervation and inhibition (Huang et al., 1999), and as noted earlier, GABAergic neuronal activity is involved in the expression of adult extinction. Hence, maternal-separation might accelerate the transition between infant-like (relapse-resistant) and adult-like (relapse-prone) extinction systems because it facilitates the development of neural processes known

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to support adult-like extinction (e.g., BDNF and GABAergic activity in the PFC).

There have been several studies showing that rats exposed to stress in adulthood exhibit impaired extinction learning and/or extinction retention (Akirav & Maroun, 2007; Garcia, Spennato, Nilsson-Todd, Moreau, & Deschaux, 2008; Izquierdo, Wellman, & Holmes, 2006; Manzanares, Isoardi, Carrer, & Molina, 2005; Miracle, Brace, Huyck, Singler, & Wellman, 2006). However, no studies to date have examined the effect of maternal-separation stress on extinction in infant rats. Of potential relevance though is the finding that stress administered early in life has been shown to accelerate developmental transitions in a fear learning paradigm. Specifically, P6-8 rats exhibit a paradoxical approach response to an odor that has been paired with shock but by P10-12 the response to a shock-paired odor switches to avoidance (Camp & Rudy, 1988; Raineki, Moriceau, & Sullivan, 2010). However, P8 rats precociously exhibit avoidance of an odor paired with shock if they were raised by a stressed mother, or were given a corticosterone (CORT) injection before test (Moriceau, Wilson, Levine, & Sullivan, 2006; Raineki et al., 2010). Thus, early life stress accelerates the transition between infant-like and adult-like responses to an odor paired with shock. Because stress has been shown to lead to an early developmental transition in odor-shock fear learning and because maternal separation has been shown to affect neural functions that support extinction learning in adult rats (Moriceau et al., 2006; Roceri et al., 2004), we asked whether maternal-separation stress would lead to the emergence of adult-like extinction learning early in development.

General Method

Subjects

Experimentally naive Sprague-Dawley derived rats, bred and housed at the School of Psychology, The University of New South Wales, were used. All experiments began when rats were 17 days of age. The day of birth was designated as P0. Only males were used, and no more than one rat per litter was used per group. Rats were housed with their mother and littermates in plastic boxes (24.5 cm long × 37 cm wide × 27 cm high) covered by a wire lid, and food and water were available ad libitum. Animals were maintained on a 12-hr light-dark cycle (lights on at 7:00 a.m.) and all behavioral testing was carried out between 8:00 a.m. and 12:00 p.m. All animals were treated according to the principals of animal care and use outlined in the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (7th ed., 2004), and all procedures were approved by the Animal Care and Ethics Committee at The University of New South Wales.

Maternal Separation

During maternal separation (MS; P2-14) all pups were removed from the home cage, weighed, and placed in an incubator for 3 hrs (starting between 9:00 a.m. and 12:00 p.m.). The ambient temperature of the incubator was maintained between 27 and 30 °C. Three centimeters of bedding was placed in the incubator so that animals could burrow down for additional warmth if required. Standard-reared (SR) animals were briefly removed from the home-cage and weighed every day during the maternal-separation period, that is,

from P2–14. There were no differences in weight between maternally separated and standard-reared animals at the end of the maternal separation period (i.e., at P14), $t_{68} = -.58$, $p = .57$.

Drug Injections

Rats in Experiment 3 were injected subcutaneously (nape of the neck) with the GABA_A partial inverse agonist FG7142 (10 mg/kg) or vehicle 10 min before testing. Injections were given at a volume of 2 ml/kg. FG7142 was suspended in 0.9% wt/vol sterile saline with one drop of Tween 80 added per 5 ml of saline. Saline with Tween added was used for the vehicle injections.

Apparatus

In Experiments 1–3, two types of chambers were used to provide different contexts. One type was a set of two identical chambers that were 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 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 shock to the floor of each chamber as required. Each chamber was housed within a separate wood cabinet so that external noise and visual stimulation were minimized. A red light-emitting diode (LED) located on the cabinet door was the sole source of illumination in these chambers. A low, constant background noise (50 dB, measured by a TENMA sound level meter, Type 72–860) was produced by ventilation fans located within the cabinet. These chambers were wiped with tap water after each experimental session. The second type was a set of two identical chambers that were rectangular (30 cm long × 30 cm wide × 23 cm high) and wholly constructed of Plexiglas, with the exception of the grid floor that was the same as in the first set of chambers. All the walls were transparent, except for the 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. Each of the chambers was housed in a separate wood cabinet so that external noise and visual stimulation was minimized. A white LED and a red LED located on the cabinet door were the sole sources of illumination in these chambers. A low constant background noise (48 dB) was produced by the ventilation fans in the cabinet. Thus, the two sets of contexts differed primarily in their size and their visual features. The CS was a white noise; noise level in the chamber was increased by 8 dB when the CS was presented. A computer controlled all presentations of the CS and the footshock US (except for Experiments 4 and 5 where the footshock US was under experimenter control). The software and hardware used were developed at The University of New South Wales.

In Experiments 1 and 2, these two sets of experimental chambers were counterbalanced. In Experiment 3, all training occurred in the first type of chamber and testing always occurred in the second type. In Experiment 4, training and testing occurred in the second type of chamber. In Experiment 5, the first type of chamber was used.

Scoring, Exclusions, and Statistics

Freezing was scored by a time sampling procedure whereby each rat was scored every 3 s as freezing or not freezing. Freezing

was defined as the absence of all movement except that required for respiration (Fanselow, 1980). These observations were then converted into a percentage score to indicate the proportion of total observations scored as freezing. A second observer, unaware of the experimental condition of each rat, scored a random sample (30%) of all rats tested. The interrater reliability was very high across all experiments ($r_s = .92$ to $.99$).

Whenever a mixed-design ANOVA was used, if the assumption of sphericity was violated, Greenhouse-Geisser adjusted p values and nominal df are reported.

In Experiments 1 and 2 significant differences in pre-CS freezing at test were detected (see Table 1 for pre-CS means at test for Experiments 1–3, and group sizes in all experiments). Because of the differences in pre-CS freezing in Experiments 1 and 2, CS-elicited freezing during test in those experiments were analyzed with ANCOVA using pre-CS freezing as a covariate. The same results were obtained, however, whether the data were analyzed with ANOVA or ANCOVA. For all analyses, a p value of $<.05$ was considered statistically significant.

Because CS-elicited freezing is difficult to detect when rats display high levels of pre-CS freezing, any rat that exhibited $>60\%$ pre-CS freezing was excluded from the final analysis. Additionally, rats that were statistical outliers at test (i.e., $>3 SD$ away from the group mean) were excluded from subsequent analysis. This resulted in the following exclusions: in Experiment 1, 1 rat from Group MS-same, 1 rat from Group SR-same, and 2 rats from Group SR-different; in Experiment 2, 1 rat from Group MS-remind, 5 rats from Group SR-remind, and 2 rats from Group

SR-not trained remind; in Experiment 3, 1 rat from Group SR-FG7142 and 1 rat from Group SR-Veh; in Experiment 4, 1 rat from Group SR-one shock. In Experiment 2, because of the large number of high baseline freezing exclusions in Group SR-remind, the data were also analyzed without exclusions using ANCOVA with pre-CS freezing as a covariate; there were no differences in the outcome of the analysis when high pre-CS freezing rats were excluded compared to when they were not. However, to ensure that only CS-elicited freezing was measured at test, and to maintain consistency with the other experiments, the reported analysis of Experiment 2 excludes the rats with high baseline freezing.

Experiment 1

Recent reports have shown that, unlike adult rats, the extinction of learned fear in P17 rats is not alleviated when animals are tested in a different context to extinction training (e.g., Gogolla et al., 2009; Kim & Richardson, 2007b; Yap & Richardson, 2007). However, early life stress has been shown to accelerate the transition between other developmental learning systems (i.e., between odor attachment/avoidance learning systems; Raineki et al., 2010). Therefore, in Experiment 1 we examined whether maternal separation would cause P17 rats to prematurely shift to the adult, relapse-prone, extinction system.

Method

The design was a 2×2 factorial, with the first factor referring to rearing condition (maternal-separation or standard-rearing) and the second referring to the context in which the animal was tested (same or different to the extinction training context). On Day 1, animals were placed in context A, and after a 2-min adaptation period, a white noise CS (8 dB above background) was presented for 10 s. The shock US (0.6 mA, 1 s) was administered during the last second of the CS. The intertrial interval (ITI) ranged from 85 to 135 s with a mean of 110 s. Six pairings of the CS and the US were given. Thirty to 60 s after the last pairing, rats were returned to their home cages. On Day 2, the animals were placed in context B and after a 2 min adaptation period presented with 30 nonreinforced presentations of the 10 s CS with a 10 s ITI. The extinction session was 12 min in duration and 30–60 s after the last CS presentation, animals were returned to their home cages. The following day, at test, the animals were placed in either the same context (B) or a different context (A) to extinction training 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. All test sessions were recorded.

Results and Discussion

CS-elicited freezing during the 5 blocks of extinction training (each block is comprised of 6 CSs) is presented in Figure 1a. As can be seen, freezing decreased across blocks, $F_{4, 144} = 44.56, p < .0001$. There was no main effect of rearing condition or testing context on within-session extinction and there were no significant interactions, largest $F = 1.9, p = .13$. Hence, rearing condition did not influence level of conditioned fear or the rate of extinction.

CS-elicited freezing at test is shown in Figure 1b. Standard-reared animals exhibited low levels of freezing at test regardless of

Table 1
Group Ns and Mean (\pm SEM) Levels of Pre-CS Freezing at Test for Maternally-Separated (MS) and Standard-Reared (SR) Groups in Experiment 1 (Renewal of Extinguished Fear), Experiment 2 (Reinstatement of Extinguished Fear), and Experiment 3 (GABA Involvement in Extinction Expression). Groups Sizes for Experiment 4 (Contextual Fear Conditioning) and Experiment 5 (Shock Sensitivity) Are Also Provided

Experiment	Groups	n	Pre-CS freezing %
1 ^a	MS-same	11	3.18 (\pm 2.48)
	MS-different	11	15.91 (\pm 5.14)
	SR-same	8	.63 (\pm .67)
	SR-different	10	10 (\pm 4.44)
2 ^a	MS-remind	10	22.93 (\pm 5.47)
	MS-no remind	11	5.89 (\pm 1.85)
	MS-not trained remind	10	3.5 (\pm 1.58)
	SR-remind	7	16.79 (\pm 7.14)
	SR-no remind	12	11.88 (\pm 4.23)
	SR-not trained remind	10	12.5 (\pm 5.34)
3	MS-FG7142	12	4.15 (\pm 2.64)
	MS-veh	11	.91 (\pm .95)
	SR-FG7142	9	.56 (\pm .59)
	SR-veh	9	.56 (\pm .59)
4	MS-1 shock	12	N/A
	MS-3 shocks	12	
	SR-1shock	10	
5	SR-3 shocks	12	
	MS	6	
	SR	6	

^a Indicates a significant difference between groups in baseline levels of freezing.

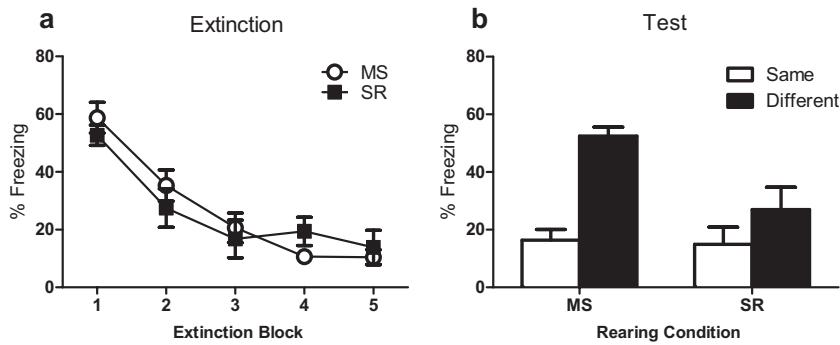


Figure 1. (a) Mean (\pm SEM) levels of CS-elicited freezing during 5 blocks (6 CS presentations in each block) of extinction training in maternally separated (MS; open-circles) and standard-reared (SR; closed-squares) rats. Regardless of rearing condition rats showed high levels of freezing at the start of extinction that decreased across blocks. (b) Mean (\pm SEM) levels of CS-elicited freezing in maternally separated (MS) and standard-reared (SR) rats tested in the same (white bars) or different (black bars) context as extinction training. High levels of fear are present in the maternally separated rats tested in a different context to extinction training.

the context (same or different to the extinction context). In contrast, levels of freezing differed according to testing context in the maternally separated animals, with those tested in a different context to extinction training exhibiting much higher levels of freezing compared to those tested in the same context as extinction training. In other words, maternally separated (MS) rats exhibited renewal at P17. The statistics confirmed this description of the data; there was a significant main effect of rearing condition, $F_{1, 35} = 4.66, p < .05$, and of test context, $F_{1, 35} = 10.10, p < .01$, as well as a significant rearing condition-by-test context interaction, $F_{1, 35} = 4.77, p < .04$. Pairwise comparisons showed that the MS group tested in the different context to extinction was freezing more than the MS group tested in the same context ($t_{20} = -5.3, p < .0001$) whereas the standard-reared (SR) animals showed low and similar freezing levels regardless of the testing context ($t_{16} = -1.4, p = .18$).

In Experiment 1 we replicated past findings showing that standard-reared P17 rats do not exhibit the renewal effect (Kim & Richardson, 2007b; Yap & Richardson, 2007). Further, and more importantly, we demonstrated that rearing animals under maternal-separation conditions resulted in the early emergence of the renewal effect. This was not because of maternal separation increasing levels of learned fear or impairing within-session extinction learning as the rearing condition of the animal did not affect those measures. This experiment is the first to demonstrate that early life stress affects relapse of extinguished fear in infant rats.

Experiment 2

The results of Experiment 1 suggest that maternal separation caused an early transition from the infant “erasure” extinction system to the mature “new learning” extinction system. Experiment 2 aimed to determine whether additional behaviors indicative of “new learning” during extinction are present in P17 maternally separated rats. In particular, in Experiment 2 we tested the hypothesis that maternally separated P17 rats, but not standard-reared P17 rats, would exhibit a return in fear after a postextinction stressor (i.e., they would exhibit reinstatement of extinguished fear).

Method

The design was a 2×3 factorial, with the first factor referring to rearing condition (maternal-separation or standard-rearing) and the second factor referring to the experimental condition (reminder group, no-reminder group, or not-trained reminder group). As in Experiment 1, animals in groups *reminder* and *no-reminder* were conditioned on P17 in context A and extinguished the following day in context B. Animals in group *not-trained reminder* were exposed to the two contexts for equivalent periods of time without being exposed to the CS or the US. The day after extinction training animals in groups *reminder* and *not-trained reminder* were placed in context B for 2 min and then given a reinstating footshock (0.4 mA, 1 s). Thirty to 60 s after the footshock animals were returned to their home cage. Animals in group *no-reminder* were exposed to the context for the same amount of time but did not receive a reinstating footshock. Twenty-four hours later all animals were tested for their fear of the CS in context B. The testing procedure was the same as that in Experiment 1.

Results and Discussion

As in Experiment 1, CS-elicited freezing decreased across blocks of extinction trials, $F_{4, 144} = 42.55, p < .0001$, as shown in Figure 2a. There was no main effect of rearing condition or testing context on within-session extinction and there were no significant interactions, largest $F = 1.49, p = .23$.

CS-elicited freezing at test is shown in Figure 2b. As can be seen, standard-reared animals exhibited low levels of freezing at test, regardless of which group they were in. Conversely, freezing in maternally separated rats differed according to group. Specifically, maternally separated rats given the reminder treatment exhibited high levels of freezing to the CS compared to MS rats not given the reminder treatment. This increase in freezing was not because of the pretest shock per se as untrained rats given the pretest shock, in both rearing conditions, exhibited low levels of freezing. In other words, the maternally separated rats exhibited clear reinstatement of extinguished fear after a postextinction

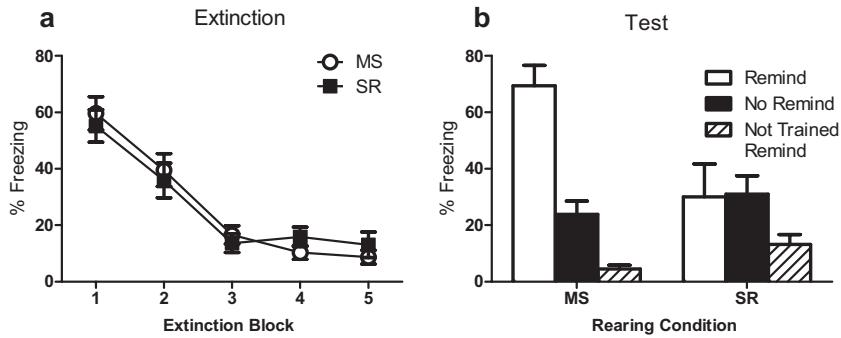


Figure 2. (a) Mean (\pm SEM) levels of CS-elicited freezing during 5 blocks (6 CS presentations in each block) of extinction training in maternally separated (MS; open-circles) and standard-reared (SR; closed-squares) rats. Regardless of rearing condition rats showed high levels of freezing at the start of extinction that decreased across blocks. (b) Mean (\pm SEM) levels of CS-elicited freezing in maternally separated (MS) and standard-reared rats (SR). There were three groups in each rearing condition: rats trained and given a postextinction reminder (white bars), rats trained and not given a reminder (black bars), and rats not trained but given the reminder (striped bars). The highest level of fear was observed in the maternally separated rats given the reminder footshock the day before test.

reminder shock whereas standard-reared animals did not. The statistics supported this description of the data. There was a significant main effect of group, $F_{2, 53} = 18.05, p < .0001$, and a significant rearing condition-by-group interaction, $F_{2, 53} = 8.52, p < .01$. Pairwise comparisons showed that group *MS-remind* displayed higher levels of freezing than group *MS-no remind*, $t_{19} = 5.69, p < .0001$, and group *MS-not trained remind*, $t_{18} = 9.38, p < .0001$. There was no difference in freezing levels between groups *SR-remind* and *SR-no remind*, $t_{17} = -.09, p = .93$, or *SR-remind* and *SR-not trained remind*, $t_{15} = 1.71, p = .11$.

In Experiment 2 we replicated past findings demonstrating that standard-reared P17 animals do not exhibit reinstatement of extinguished fear (Kim & Richardson, 2007a). However, the finding of interest was that reinstatement of extinguished fear did occur after a postextinction footshock stress if animals had been exposed to maternal separation early in development. These data are consistent with the findings from Experiment 1 that showed that maternally separated animals also exhibit renewal of extinguished fear at P17. Hence, it appears that depriving animals of maternal care early in development results in the early emergence of adult-like extinction learning, which leads to a greater propensity for relapse of extinguished fear at P17.

Experiment 3

Consistent with past research, Experiments 1 and 2 showed that context- and stress-mediated relapse was not observed after extinction in P17 standard-reared rats. Further, and more importantly, these experiments demonstrated that maternally separated P17 rats exhibited context- and stress-mediated relapse after extinction training. In other words, maternally separated animals exhibited adult-like extinction at an earlier stage of development. Thus, in Experiment 3 we examined whether a neurotransmitter system involved in adult extinction, namely the GABAergic system, would also be involved in extinction at P17 after maternal separation.

In adult rats, the expression of extinction is thought to involve an active process of GABA-mediated fear inhibition. Hence, when GABAergic inhibition is reduced at test, via an injection of the GABA_A partial inverse agonist FG7142, animals return to a fearful state; that is, extinction is not expressed (Harris & Westbrook, 1998). A pretest injection of FG7142 has been shown to have no effect on fear relapse in P17 rats (Kim & Richardson, 2007b). This experiment examined whether pretest administration of FG7142 caused a return of fear in P17 rats if they had been exposed to maternal-separation early in life.

Method

The design was a 2×2 factorial, with the first factor referring to rearing condition (maternal-separation or standard-rearing) and the second referring to the drug administered at test (FG7142 or Vehicle). As in Experiment 1, all rats were trained on P17 and were extinguished for their fear of the CS the following day. Testing occurred 24 hr later, in the same context as extinction training. Ten minutes before the test, half the animals from each rearing condition were injected with the GABA partial inverse agonist FG7142. The remaining animals from each rearing condition were injected with vehicle.

Results and Discussion

As in Experiments 1 and 2, CS-elicited freezing decreased across the 5 extinction blocks, $F_{4, 148} = 57.99, p < .0001$, as shown in Figure 3a. No other main effects or interactions were significant (largest $F = 1.16, p = .33$). Thus, again, rearing condition had no effect on the level of conditioned fear or the rate of extinction.

There was a differential effect of the drug on CS-elicited freezing at test depending on the rearing condition of the animal. Specifically, maternally separated rats given FG7142 before test exhibited much higher levels of CS-elicited freezing than the maternally separated animals that were given Vehicle before test.

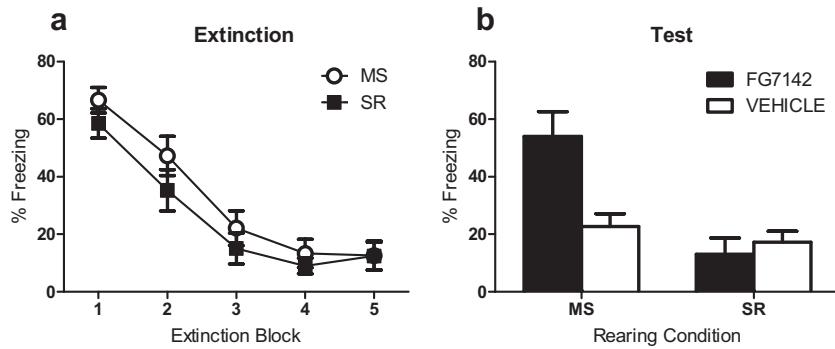


Figure 3. (a) Mean (\pm SEM) levels of CS-elicited freezing during 5 blocks (6 CS presentations in each block) of extinction training in maternally separated (MS; open-circles) and standard-reared (SR; closed-squares) rats. Regardless of rearing condition rats showed high levels of freezing at the start of extinction that decreased across blocks. Figure 3. (b) Mean (\pm SEM) levels of CS-elicited freezing at test in maternally separated (MS) and standard-reared (SR) rats given the GABA_A partial inverse agonist FG7142 (black bars) or vehicle (white bars) 10 min before test.

In contrast, there was no difference in freezing levels between the SR animals that were given FG7142 or Vehicle before test. In other words, GABAergic inhibition was involved in the expression of extinction at P17 only in the maternally separated animals. The statistics confirmed this description of the data. There was a significant main effect of rearing condition, $F_{1, 37} = 13.95, p < .01$, of test drug, $F_{1, 37} = 5.06, p < .04$, and a significant rearing condition-by-test drug interaction, $F_{1, 37} = 8.05, p < .01$ (Figure 3b). Post hoc *t*-tests showed that FG7142 resulted in higher freezing levels at test than did Vehicle injection in the maternally separated animals, $t_{21} = 3.27, p < .01$, but not in the standard-reared animals, $t_{16} = -.56, p = .59$.

In Experiment 3 we replicated past research showing that FG7142 administration has no effect on CS-elicited freezing after extinction in standard-reared P17 animals (Kim & Richardson, 2007b). Further, and more importantly, we also demonstrated that injection of FG7142 enhanced CS-elicited freezing after extinction in maternally separated rats. These data suggest that GABAergic inhibition is involved in the expression of extinction at P17 only in animals that had been exposed to a stressful rearing environment. Taken together, the results from Experiments 1–3 provide clear evidence that maternal-separation leads to a fundamental change in the system supporting extinction early in development. More specifically, after maternal-separation, P17 rats exhibit adult-like extinction learning.

Experiment 4

Given that maternally separated P17 animals show more adult-like extinction than standard-reared animals of the same age, it is possible that other forms of learning in P17 rats are more adult-like after maternal separation. For example, relative to adult animals, P17 rats exhibit impaired formation of long-term memory (LTM) for a simple context-shock association (Rudy, 1993). In Experiments 1 and 2, P17 animals that were exposed to maternal separation were able to use contextual information in a sophisticated way to modulate the expression of extinction memories. Hence, it was hypothesized that maternal separation would also enhance the ability of P17 rats to remember simple context-shock associations.

Method

The design was a 2×2 factorial, with the first factor referring to rearing condition (maternal-separation or standard-rearing) and the second referring to the number of training shocks (one or three). On Day 1, P17 rats were placed in the training context and after 2 min given either 1 or 3 footshocks spaced 30 s apart. Ten seconds after the last footshock animals were returned to their home cages. At test, 24 hr later, rats were placed in the same context and their freezing was scored for 3 min.

Results and Discussion

Animals given one shock in the context exhibited low freezing levels at test the following day, regardless of rearing condition. However, when the number of footshocks was increased to three, rats in both groups showed an increase in context freezing. This increase was much larger in the maternally separated animals. The statistics confirmed this description of the data. There was a significant main effect of rearing condition, $F_{1, 42} = 8.51, p < .01$, and of number of footshocks, $F_{1, 42} = 31.38, p < .0001$. Further, there was a significant rearing condition-by-number of footshocks interaction, $F_{1, 42} = 8.84, p < .01$ (see Figure 4). Pairwise comparisons showed that the maternally separated animals exhibited greater context fear than standard-reared animals after 3 footshocks, $t_{22} = 3.24, p < .01$, but not after 1 footshock, $t_{20} = -.09, p = .93$.

In Experiment 4, we replicated the finding that standard-reared P17 rats exhibit poor LTM for simple context-shock associations (Rudy, 1993). However, we showed that LTM for context-shock associations was enhanced after maternal-separation rearing. Interestingly, the facilitative effect of maternal-separation on context learning was only apparent when animals received multiple footshocks in the context. This could indicate that a basal level of learning needs to be expressed in standard-reared rats before the augmenting effects of maternal-separation on LTM for contexts can be detected. Alternatively, it may be the case that the parameters were not sensitive enough to detect a difference between maternally separated and standard-reared animals after a single footshock.

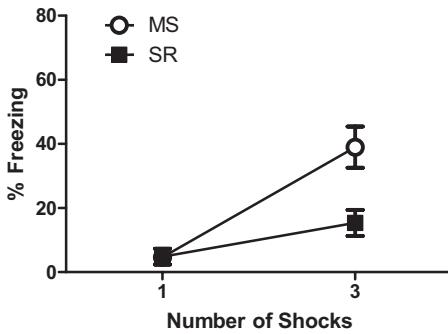


Figure 4. Mean (\pm SEM) levels of freezing to the context in maternally separated (MS; open-circles) and standard-reared (SR; closed-squares) rats at test. During training rats received either 1 or 3 footshocks. Rats from both rearing conditions show higher levels of freezing to the context after 3 footshocks than after 1 footshock. Maternally separated rats show a greater level of freezing to the context than do the standard-reared rats after 3 footshocks.

Experiment 5

Experiments 1–4 showed that maternally separated rats are more prone to exhibit fear relapse after extinction at P17 and have better LTM for a shock-associated context. These results are likely because of maternal separation accelerating the functional development of neural structures and neurotransmitter systems involved in extinction and context learning. An alternative possibility, however, is that maternal separation causes rats to become more sensitive to footshock—leading to greater conditioned fear and more relapse. Thus, Experiment 5 tested shock sensitivity in maternally separated and standard-reared rats.

Method

Rats were placed in the experimental chamber and after 20 s were exposed to a maximum of 32 footshocks (each 1 s in duration) increasing in intensity from 0.15 to 0.3 mA. Each shock was separated by 3 s and the intensity increased by 0.01 mA every second shock. The thresholds for three behavioral responses to shock were recorded by an observer who was blind to the rearing condition of the animals. *Flinching* was defined as a sudden, brief muscle contraction that did not involve locomotion. *Shuffling* was defined as a more pronounced movement involving locomotion. *Vocalization* was defined as an audible vocalization made by the animal upon presentation of the footshock. Thresholds were defined as the lowest shock intensity at which the response was exhibited. Testing was terminated when the thresholds for all three responses were determined. The data were analyzed as a two-group repeated measures design where the two groups referred to the rearing condition of the animals (maternal-separation or standard-rearing) and the repeated measure was the threshold for each behavioral response to shock (flinching, shuffling, and vocalization).

Results and Discussion

Rearing condition had no effect on footshock sensitivity in P17 rats. There was a significant effect of behavior, $F_{2,20} = 12.62, p <$

.0001, where the threshold was lowest for flinch, intermediate for vocalization, and highest for shuffle. No other main effects or interactions were significant (largest $F < 1$). See Table 2 for mean (\pm SEM) threshold for each behavioral response to shock.

General Discussion

The results of this study demonstrate that a potent stressor applied in the early postnatal period, maternal-separation, results in a precocious transition from the infant, relapse-resistant, “erasure” extinction system to the adult, relapse-prone, “new learning” extinction system. This early developmental switch resulted in premature emergence of stress- and context-mediated relapse following extinction learning, as well as early integration of GABAergic inhibition in the expression of extinction. Additionally, our results show that maternal-separation enhanced LTM for a context in P17 rats. Finally, these effects were not the result of maternal-separation increasing the rat’s sensitivity to footshock.

A number of recent studies have examined the occurrence of relapse behaviors in standard-reared P17 animals (for review see Kim & Richardson, 2010). In all of those studies, extinction at P17 was found to result in a robust reduction in fear, which was resistant to relapse. Indeed, it has been suggested that the fear reduction exhibited by P17 animals during extinction may actually reflect the erasure of fear memories (Gogolla et al., 2009; Kim & Richardson, 2008). In the current study, we replicated the finding that standard-reared P17 animals use an erasure-like mechanism during fear extinction—resulting in low relapse of extinguished fear. Further, and more importantly, we showed that a very simple early life environmental manipulation (maternal-separation) caused a dramatic shift in the mechanism by which P17 rats extinguished fear. Specifically, the occurrence of context-, stress-, and GABA-mediated fear relapse in maternally separated P17 animals suggests that maternal-separation caused an early developmental switch from an erasure-like (relapse-resistant) extinction system to a new-learning (relapse-prone) extinction system. Further, this manipulation also led to enhanced LTM for a shocked context at P17. Although the exact mechanisms supporting the observed early developmental shift in extinction learning and context memory are presently unknown, one hypothesis is that maternal separation may accelerate the development of the brain structures critical for these functions (i.e., the amygdala, PFC, and hippocampus; but see Rudy, 2009 for limitations to hippocampal involvement in context conditioning). Indeed, maturation of these structures, both at the cellular and systems level, coincides with termination of the critical period for erasure-like extinction and the development of adult-like contextual fear memory (Bronzino, Abu-Hasaballah, Austin-LaFrance, & Morgane, 1994; Gogolla et al., 2009; Rudy, 1993; VanEden & Uylings, 1985). Hence, it is

Table 2
Mean (\pm SEM) Thresholds (mA) for Shock-Elicited Flinching, Vocalization, and Shuffling in Maternally-Separated and Standard-Reared Rats

Group	Flinching	Vocalization	Shuffling
Maternally separated	0.16 (.01)	0.18 (.00)	0.23 (.02)
Standard reared	0.17 (.00)	0.20 (.02)	0.22 (.02)

possible that by accelerating the maturation of those regions required for flexible fear responding, stress prepares young animals at risk to behave more cautiously toward ambiguous cues and fear signals in the environment (i.e., to exhibit stronger context fear and greater relapse of extinguished fear).

One alternative explanation for our results is that handling cues alone may have been sufficient to cause the early transition in fear and extinction learning seen in maternally separated animals. Indeed, previous studies have shown that P18–30 animals, handled for 15 min per day from P1–15, have better LTM for a context associated with a footshock (Beane, Cole, Spencer, & Rudy, 2002). However, in our experiments, standard-reared animals were exposed to the same handling cues as maternally separated animals. However, the standard-reared animals did not exhibit the early transition in fear and extinction learning. In fact, the low levels of freezing seen in our standard-reared animals following contextual conditioning and extinction training are consistent with previous data using nonhandled P17 rats (e.g., Kim & Richardson, 2007a, 2007b; Rudy, 1993). Hence, it is unlikely that the enhanced performance seen in our maternally separated animals can be attributed to the handling they experienced.

Previous studies have also shown that different early life conditions can affect the emergence of adult-like fear and extinction learning. For example, environmental enrichment has been shown to enhance LTM for a context in P18 rats (Woodcock & Richardson, 2000). In addition, chronic injections of fibroblast growth factor-2 (FGF2) across early postnatal life (P1–5) have been shown to enhance LTM for contexts and to increase context-mediated relapse of extinguished fear in P17 rats (Graham & Richardson, 2010). It may be that similar mechanism(s) mediate the enhancement of long-term contextual memory and emergence of adult-like extinction in P17 rats exposed to these various early life manipulations. Interestingly, in a previous study (that used the same maternal separation paradigm used here) it was found that maternal-separation caused a transient increase in BDNF mRNA in the hippocampus and PFC of P17 rats (Roceri et al., 2004). BDNF facilitates hippocampal long-term potentiation and enhances spatial learning in mice (Linnarsson, Björklund, & Ernfors, 1997). Further, transgenic mice overexpressing BDNF show accelerated development of visual acuity and intracortical GABA-mediated inhibition (see Berardi, Pizzorusso, Ratto, & Maffei, 2003). Hence, the contextual-modulation of extinction, enhanced contextual long-term memory, and early integration of GABAergic inhibition in extinction expression seen in maternally separated P17 rats may have been mediated by a BDNF-dependent mechanism. If this is correct, then we might expect to see impairment in contextual learning abilities in adult maternally separated rats as these animals have been shown to have lower hippocampal BDNF mRNA levels (Roceri et al., 2004). Indeed, in a recent study that examined this issue, acquisition of a context-shock association was found to be slower in adult rats exposed to maternal-separation early in life (Guizarro et al., 2007).

The fact that maternally separated young rats are more prone to fear relapse following extinction training is of potential relevance to the treatment of anxiety in young persons. Anxiety disorders are one of the most common mental disorders, with 29% of the population in the United States meeting *DSM-IV* criteria for any anxiety disorder at some point in their lives (Kessler et al., 2005). Considering the high comorbidity between psychiatric disorders

and adverse early life environments (e.g., Repetti, Taylor, & Seeman, 2002), one potential route to anxiety is through the effects of stress on the developing brain. Although there is a large body of research examining the long-term consequences of early life stress on the adult brain (e.g., Andersen & Teicher, 2004; Ladd, Huot, Thrivikraman, Nemeroff, & Plotsky, 2004; Roth, Lubin, Funk, & Sweatt, 2009), the early life outcomes of stress exposure have only recently begun to receive the same attention. As many anxiety disorders manifest early in life (Cartwright-Hatton, McNicol, & Doubleday, 2006; Cicchetti & Toth, 2005; Newman et al., 1996), focusing on the interaction between early life stress and early life fear inhibition could be of significant value. Indeed, such a developmental focus may facilitate the development of more effective treatments for early life anxiety (McEwen, 2000; Pine, Helfinstein, Bar-Haim, Nelson, & Fox, 2009). Importantly, in a recent study that looked at developmental outcomes of chronic stress, previously institutionalized children were shown to exhibit a more adult-like pattern of amygdala activity to fearful over neutral faces than a sample of nonstressed peers, and this precocious pattern of responding was associated with poorer social competence (Tottenham et al., in press). Hence, it is possible that early life stress may hasten limbic system development leading to adverse extinction behavior and poor social development across species.

This study is the first attempt, to our knowledge, to understand how the early life environment of an animal influences its propensity for fear relapse after extinction. Importantly, we have shown that the chronological age of the animal is not the only factor that determines the occurrence of relapse behavior following extinction. Rather, the present research shows that the rearing environment of an animal is a critical factor that determines whether relapse of extinguished fear will be expressed or not. We found that a stressful rearing environment led to offspring that exhibited context-, stress-, and GABA-mediated relapse of extinguished fear at an earlier age than their nonstressed contemporaries. Further, these animals were more prepared to learn about an aversive context. Hence, in addition to early life adversity acting as a potent risk factor for the development of anxiety disorders in young people (Repetti et al., 2002), our research suggests that these young people may be more likely to exhibit relapse after exposure-based treatment for anxiety than their same-aged, nonstressed peers.

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