Research Report

Infant Stress Exposure Produces Persistent Enhancement of Fear Learning Across Development

ABSTRACT: In recent years, it has become increasingly clear that early life stress experiences persistently impact subsequent physiological, cognitive, and emotional responses. In cases of trauma, these early experiences can result in anxiety disorders such as phobias and posttraumatic stress disorder. In the present paper, we examined the effects of infant footshock stress exposure at postnatal day (PND) 17 on subsequent contextual fear conditioning at postnatal days 18 (Experiment 1), 24 (Experiment 2), or 90 (Experiment 3). In each experiment, PND17 footshock stress exposure enhanced later fear conditioning, indicating that the stress enhancement of fear learning (SEFL) persists throughout development. Memory for the original stress exposure context was gradually forgotten, with significant fear expression evident at PND20, and a complete lack of fear expression in that same context at PND90. These data suggest that the stress-enhancing component of infant fear learning is dissociable from the infant contextual fear memory per se. In other words, early life stress produces persistent effects on subsequent cognition that are independent of the memory for that early life event. © 2013 Wiley Periodicals, Inc. Dev Psychobiol

Keywords: rat; SEFL; infantile amnesia; forgetting; early-life stress; fear conditioning

INTRODUCTION

A growing number of research studies is drawing attention to, and emphasizing the important influence of, early-life adversity on later cognitive and behavioral outcomes. Furthermore, manipulations of stress or trauma across the developmental lifespan from infancy through adulthood have very different effects depending on both the age at initial stress exposure as well as the age at testing (Jankord et al., 2011; Kosten, Kim, & Lee, 2012; Lupien, McEwen, Gunnar, & Heim, 2009; Moriceau, Raineki, Holman, Holman, & Sullivan, 2009; Toledo-Rodriguez & Sandi, 2007). Adversity produces enduring physiological effects involving the hypothalamic–pituitary–adrenal (HPA) stress response system that, in turn, modifies an organism’s sensitivity to future threat (e.g., Alexander et al., 2012; Hunter, Minnis, & Wilson, 2011; Morley-Fletcher, Rea, MacCari, & Laviola, 2003).

A variety of animal models have been developed to examine the effects of stressful experiences on subsequent cognition, behavior, and emotional reactivity (e.g., Farrell, Senglaub, & Wellman, 2013; Hill, Hellmans, Verma, Gorzalka, & Weinberg, 2012; Kosten et al., 2012; Nishi, Hori-Hayashi, Sasagawa, & Matsunaga, 2013; Shors & Servatius, 1997; Shors et al., 1992). Fear conditioning provides an ideal model for a number of reasons. First, it incorporates both stress and learning components that are well characterized at the neural level and lend themselves to the analysis of physiological changes corresponding to altered behavioral responses. Fear and its neural circuitry are highly conserved across species, providing a valid animal model for fear-related human conditions (Dias, Banejee, Goodman, & Ressler, 2013). In addition, fear memories acquired during adulthood can be reliably
measured and are very stable, remaining intact for at least 16 months following conditioning (Gale et al., 2004; Quinn, Ma, Tinsley, Koch, & Fanselow, 2008). Finally, fear conditioning is produced within a single session, allowing for strict control over the developmental timing of the experience (Hunt et al., 2007).

Learned fear responses can be observed throughout much of the rodent lifespan. However, important conditioned response (CR)-specific dissociations are evidenced in pre-weanling rats. Conditioned freezing emerges at a younger age than conditioned cardiovascular responses, which emerge prior to conditioned fear potentiation of startle (Hunt & Campbell, 1997; Hunt, Hess, & Campbell, 1998). Richardson and coworkers (Richardson & Fan, 2002; Yap, Stapinski, & Richardson, 2005) have shown that fear memory expression is appropriate to the rat’s age at training, rather than testing. Thus, if animals are trained at an age when they are unable to express freezing and tested at an age when freezing can be expressed, the animals will not show the freezing response. In addition, sensory-specific differences are evident during early development; conditioned stimulus (CS) modality determines the age at which fear CRs are expressed (Hunt & Campbell, 1997; Richardson, Paxinos, & Lee, 2000; Richardson, Wang, & Campbell, 1995; Rudy, Vogt, & Hyson, 1984). Fear conditioning to multimodal contextual CSs appears to emerge later than conditioning to discrete auditory CSs (Rudy, 1991, 1993; Rudy & Morledge, 1994). Juvenile rats fear conditioned at postnatal day 18 (PND18) and tested 24 hr later show little evidence of context conditioning compared with rats fear conditioned at PND23 (Rudy & Morledge, 1994). It is important to note, however, that when infant rats are conditioned using multiple footshocks (or higher footshock intensity), substantial fear conditioning may be observed at earlier ages (McKinzie, Chen, & Spear, 1998; Pisano, Ferreras, Krapacher, Paglini, & Arias, 2012). Similarly, early postnatal stress in the form of maternal separation or maternal corticosterone exposure induces a premature ability to acquire fear memories in infancy (Callaghan & Richardson, 2012; Moriceau, Shionoya, Jakubs, & Sullivan, 2009).

In adult rats, exposure to a significant stressor prior to fear conditioning can produce a disproportionately enhanced fear response. Specifically, exposure to an initial stressor (4 or 15 unsignaled footshocks) dramatically increases subsequent fear conditioning to a novel context or discrete tone CS (Long & Fanselow, 2012; Ponomarev, Rau, Eger, Harris, & Fanselow, 2010; Rau, DeCola, & Fanselow, 2005), and is not a result of context generalization. However, exposure to only a single unsignaled footshock does not produce the subsequent enhancement of fear learning, suggesting that a significant activation of stress response circuits is necessary for stress enhancement of fear learning (SEFL) to occur (Rau & Fanselow, 2009).

Because the age at stress exposure and age at testing are so important to behavioral outcomes, a more systematic examination of the SEFL effect across development is needed. The present experiment assessed whether infant footshock stress exposure on PND17 enhances subsequent contextual fear conditioning across development (PND18, PND24, PND90). In addition, this experiment evaluates the persistence of memory for the original context in which the infant stress experience occurred.

**METHODS**

**Subjects**

One hundred seven Long Evans rats, bred and housed at Miami University, were used in this experiment (breeders supplied by Harlan Laboratories, Indianapolis, IN). Litters were culled to 10 pups (5 male, 5 female whenever possible) on PND4 or PND5 (date of birth is PND0). Rats were weaned on PND21 and group housed with same-sex littermates. In each experiment, rats were assigned to one of four conditions. No more than two males and two females from a single litter were assigned to a particular experimental condition (sampled from a total of 14 litters). Where there were same-sex duplicates from a given litter within an experimental condition, data were averaged to provide a single value. Rats were housed in plastic cages throughout the experiments. Animals were maintained on a 12:12-hr light/dark cycle (lights on at 6:00 a.m.), with experiments performed during the light cycle. Food and water were provided ad libitum in the home cage throughout the experiments. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition, National Academies Press, Washington, D.C., 2011) and were approved by the Miami University Institutional Animal Care and Use Committee.

**Apparatus**

**Context A.** Stress exposure was performed in four identical conditioning chambers (32.4 × 25.4 × 21.6 cm³; Med-Associates, Inc., Georgia, VT) housed within sound-attenuating cubicles. Chambers consisted of a white plastic back wall, aluminum sidewalls, and clear Plexiglas ceiling and front door. They were brightly lit (1401ux) and contained flat, stainless steel grid floors with underlying pans containing approximately 10 ml of 50% vanilla solution (Meijer Distribution, Inc., Tipp City, OH). Odorless sodium hydroxide (5%) was used to clean each chamber prior to the placement of each rat. Grid floors were wired to a shock generator and scrambler (Med-Associates, Inc.).

**Context B.** In a separate room, four similar chambers were used for fear conditioning. These chambers contained black...
Plexiglas triangular inserts and the floor was composed of 18-staggered stainless steel rods (two rows, .5 cm vertically apart; in each row, each rod was 1.5 cm apart). These chambers were completely dark and near-infrared lighting was used for video recording. Boxes were cleaned using a 5% acetic acid solution. This solution was also placed in pans underneath the grid floors in order to provide a context odor (approx. 10 ml/pan).

**Video Behavioral Recording.** Rats were continuously monitored throughout all behavioral sessions using progressive scan video cameras with visible light filters (VID-CAM-MONO-2A; Med Associates, Inc.). These cameras were connected to a computer running Video Freeze software (Med Associates, Inc.) designed for automated assessment of motion, including defensive freezing (Anagnostaras et al., 2010).

**Procedure**

**Stress Exposure (Context A).** Stress exposure took place on PND17 in Context A for all animals. Prior to the stress exposure rats were transported in their home cages to a brightly lit area (250 lux) outside of the behavioral testing suite. A clear plastic cage was used to transport each rat to the behavioral testing room. In the 15-shock stress exposure group, footshock (1 mA, 1 s) was delivered beginning 180 s following placement in the chamber. Additional shocks were delivered according to a variable inter-shock interval of 240–480 s. Total session duration was 93 min. No-shock animals were placed into the chambers for an equivalent duration in the absence of footshock. Following stress exposure, rats were transported back to their home cages and returned to the animal housing facility.

**Fear Conditioning (Context B).** Fear conditioning took place in Context B on PND18 (Experiment 1; Fig. 1a), PND24 (Experiment 2; Fig. 2a), or PND90 (Experiment 3; Fig. 3a). To reduce generalization between contexts, a novel method was used for transportation to Context B. Rats were transported in their home cages to a dark room (0 lux) prior to testing. Small, plastic black boxes (18 × 32 × 9 cm³) were then used to transport rats into Context B. Rats received zero or one footshock (1 mA, 1 s) 180 s following placement in the chamber, and were removed from the chamber 30 s following termination of shock. Baseline freezing during the first 180 s in this novel context was assessed and used as a measure of generalization between the stress exposure context (Context A) and the fear conditioning context (Context B). Following fear conditioning, rats were transported back to their home cages in the black transport boxes as before.

**Context Tests.** Fear memory for Context B was tested first, 1 day following fear conditioning. Rats were transported to Context B as before. They were placed into the fear conditioning chambers and their behavior was recorded for an 8 min shock-free period. Rats were then returned to their home cages. The next day, rats were transported to Context A as they were previously, for a similar 8 min shock-free test session.

![FIGURE 1](a) Schematic of procedures used in Experiment 1. (b) Baseline percentage of time spent freezing (±SEM) on PND18 during the fear conditioning session in Context B. (c) Percentage of time spent freezing (±SEM) during the test session on PND 19 in the fear conditioning context (Context B). (d) Percentage of time spent freezing (±SEM) during the test session on PND 20 in the original training context (Context A). Number of observations per group were: no shock/no shock = 9; no shock/1 shock = 9; 15 shocks/no shock = 8; 15 shocks/1 shock = 10.
Data Analysis

The percentage of time spent freezing, defined as an absence of movement except that necessary for respiration (Fanselow, 1980), was used as the dependent measure of fear responding. Freezing was calculated as a percentage of time using Video Freeze software (Med-Associates, Inc.). Baseline freezing was measured during the first three minutes of the fear conditioning session in novel Context B (prior to footshock). Freezing as a result of fear conditioning (in Context B) and in response to the stress exposure context (Context A) was measured during the full 8 min of the corresponding context test sessions. All statistics were calculated using SPSS version 17.0. Factorial analyses of variance (ANOVAs; factors: stress exposure and fear conditioning) were conducted separately for each experiment to analyze freezing during the baseline, Context A test, and Context B test periods, with critical value $\alpha = .05$. A priori planned comparisons between groups were performed using Fisher’s LSD with $\alpha = .05$.

RESULTS

Experiment 1: PND17 Footshock Stress Exposure Enhances Fear Conditioning on PND18

Baseline Freezing. Baseline freezing during the first exposure to Context B (fear conditioning) was low across all groups (Fig. 1b). Nonetheless, there was a significant main effect of stress exposure [$F(1,32) = 11.49$, $p < .01$]. The 15-shock groups froze significantly more than the no-shock groups (in Context B). This suggests that some generalization between contexts occurs at this age (initial stress exposure occurred in Context A). There was no significant difference in baseline freezing between the groups assigned to receive zero versus one footshock during the fear conditioning session.

Context B Test. During the Context B test (i.e., fear conditioning context), there was a significant main effect of stress exposure [$F(1,32) = 7.24$, $p < .05$], but no main effect of fear conditioning and no interaction between these factors (Fig. 1c). A priori planned comparisons revealed that among rats fear conditioned on PND18, prior 15-shock stress exposure resulted in significantly greater context fear conditioning compared with rats that received no stress exposure ($p < .05$). Further, among rats that did not receive fear conditioning in Context B on PND18, prior stress exposure had no effect on freezing.

Context A Test. When subsequently tested in Context A (i.e., original stress exposure context), there was a

FIGURE 2  (a) Schematic of procedures used in Experiment 2. (b) Baseline percentage of time spent freezing (±SEM) on PND24 during the fear conditioning session in Context B. (c) Percentage of time spent freezing (±SEM) during the test session on PND 25 in the fear conditioning context (Context B). (d) Percentage of time spent freezing (±SEM) during the test session on PND 26 in the original training context (Context A). Number of observations per group were: no shock/no shock = 7; no shock/1 shock = 6; 15 shocks/no shock = 5; 15 shocks/1 shock = 5.
significant main effect of stress exposure \(F(1,32) = 31.14, p < .001\), but no main effect of fear conditioning and no interaction between these factors (Fig. 1d). Rats that had received 15 footshocks showed significantly greater freezing to that context (Context A) compared to rats that had not received stress exposure, independent of subsequent fear conditioning in Context B.

Experiment 2: PND17 Footshock Stress Exposure Enhances Fear Conditioning on PND24

**Baseline Freezing.** Baseline freezing during the first exposure to Context B (fear conditioning) was low across all groups, with no significant differences resulting from prior stress exposure (Fig. 2b); therefore, there was no apparent context generalization at this age. Additionally, no differences in baseline freezing were observed between groups assigned to receive zero versus one footshock during fear conditioning.

**Context B Test.** During the Context B test, there was a significant main effect of stress exposure \(F(1,19) = 23.06, p < .001\) as well as a significant main effect of fear conditioning \(F(1,19) = 47.85, p < .001\), but no interaction (Fig. 2c). A priori planned comparisons revealed that among rats fear conditioned on PND24, prior stress exposure resulted in significantly greater context fear conditioning \((p < .01)\). Among rats that did not receive fear conditioning in Context B on PND24, prior stress exposure significantly increased freezing during this context test. This suggests that, in this experiment, the apparent stress enhancement in fear learning among the fear conditioned animals may be, in part, due to non-specific conditioning effects (e.g., context generalization). However, it is unlikely that these non-specific effects explain the observance of SEFL in these experiments, since they are not seen in Experiments 1 or 3.

**Context A Test.** Testing in Context A yielded significant main effects of stress exposure \(F(1,19) = 26.74, p < .001\) and fear conditioning \(F(1,19) = 5.34, p < .05\) as well as an interaction between these factors \(F(1,19) = 5.52, p < .05\) (Fig. 2d). Rats that had received 15 footshocks showed significantly greater freezing to that context compared to rats that had not received stress exposure. However, if these stress-exposed rats also received fear conditioning in Context B, they showed significantly greater freezing when returned to the stress exposure context (Context A) later, as if fear conditioning in Context B served as a reminder of the prior stress exposure.
Experiment 3: PND17 Footshock Stress Exposure Enhances Fear Conditioning on PND90

Baseline Freezing. Baseline freezing during the first exposure to Context B (fear conditioning) was low across all groups (Fig. 3b). As with rats fear conditioned on PND24, there were no significant differences resulting from prior stress exposure. Again, no differences in baseline freezing were observed between groups assigned to receive zero versus one footshock during fear conditioning.

Context B Test. During the Context B test, there was a significant main effect of stress exposure \( F(1,20) = 5.21, p < .05 \), a significant main effect of fear conditioning \( F(1,20) = 24.99, p < .001 \), and a significant interaction \( F(1,20) = 5.03, p < .05 \) (Fig. 3c). A priori planned comparisons revealed that among rats fear conditioned on PND90, prior stress exposure resulted in significantly greater context fear conditioning \( (p < .01) \). Further, among rats that did not receive fear conditioning in Context B on PND90, prior stress exposure had no effect on freezing.

Context A Test. Testing in Context A yielded no significant main effect of stress exposure, no main effect of fear conditioning and no interaction (Fig. 3d), with all groups freezing at very low levels \(<5\%\). This shows that, compared with other age groups (PND18 and PND24), adult rats no longer show fear responding in Context A. This indicates that the rats have forgotten the context in which the initial stress experience occurred on PND17.

DISCUSSION

The present data show that exposure to a significant stressor during infancy (PND17) leads to the subsequent enhancement of fear learning in infants (PND18), juveniles (PND24), and adults (PND90). This is consistent with previous studies showing that prior stress exposure in adulthood facilitates later fear conditioning (e.g., Rau et al., 2005; Rau & Fanselow, 2009). In adults, this stress enhancement extends beyond contextual fear conditioning. When rats are exposed to a significant 15-footshock stressor, subsequent conditioning to a tone that is paired with a single footshock is enhanced (Rau et al., 2005). This suggests that the mechanism mediating the stress enhancement effect may, in part, involve amygdala hyperexcitability since both context and tone fear conditioning are dependent upon the amygdala. Consistent with this possibility in the context of early life stress exposure, Richardson and coworkers have shown that early fear conditioning (PND16 or PND23) leads to persistent mitogen-activated protein kinase (MAPK) activity in the amygdala, though no one has looked at whether this increased activity persists into adulthood (Kim, Li, Hamlin, McNally, & Richardson, 2012). Further, in adult rats, long-lasting effects of 15-footshock stress exposure on amygdalar gene expression have been observed (Ponomarev et al., 2010), reflecting functional and structural changes in neurons and astroglia that are necessary for plasticity and the SEFL.

At 17–18 days of age, rats demonstrated significant context fear conditioning with 15 footshocks (Fig. 1d), but no context fear conditioning with 1 footshock (Fig. 1c). However, by 24 days of age, rats show significant context fear conditioning with a single footshock (Fig. 2c). This is consistent with previous studies showing weaker retention of contextual fear conditioning for infant rats compared to juvenile and adult rats (Hunt et al., 2007; Rudy, 1991, 1993; Rudy & Morledge, 1994; Schiffino, Murawski, Rosen, & Stanton, 2011). It is important to note, however, that while PND17 rats do not show significant freezing to a context associated with presentation of a single footshock, more subtle indications of memory for the fear conditioning experience are present in these infant rats (Pisano et al., 2012). Further, with an increase in footshock intensity or the number of footshocks, significant fear conditioning can be observed in PND17 rats (McKinzie et al., 1998). Such increases in the magnitude of the footshock, as with our 15-footshock exposure, yields significant context fear conditioning that is consistent with data showing that early postnatal stress (maternal separation; maternal corticosterone exposure) induces a premature ability to acquire fear memories (Callaghan & Richardson, 2012).

Conditioning to the 15-footshock context on PND17 is remembered well when tested on PND20 (Fig. 1d). However, gradual forgetting is observed with a complete absence of fear responding observed by PND92 (see Figs. 2d and 3d). Previous studies have shown that infant rats experience rapid forgetting of fear conditioning within 10 days of acquisition (e.g., Kim, McNally, & Richardson, 2006; Weber, McNally, & Richardson, 2006). However, it is important to recognize that despite the apparent forgetting of infant fear memories in the present experiments, this early life stress experience had a lasting impact on fear learning in adulthood (Fig. 3c). These data add to the growing literature on how early life experiences alter learning later in life (e.g., Sevelinges et al., 2007). There are at least two possible, yet not mutually exclusive, explanations for these findings. First, it is possible that the infant context fear memory is completely forgotten and the early life
stress experience alters the state of the animal such that future stress reactivity (and learning about future threats) is enhanced (e.g., Bilbo & Schwarz, 2012; Campbell & Spear, 1972; Matsumoto, Yoshioka, & Togashi, 2009; Ponomarev et al., 2010). Thus, early life stress produces persistent effects on subsequent cognition that are independent of the memory for that early life event. Alternatively, the infant contextual fear memory may persist, despite apparent forgetting at the behavioral level. For example, Li and Richardson (2013) recently showed that infant fear conditioning renders subsequent fear conditioning NMDA receptor-independent, despite complete forgetting of the infant memory at the behavioral level. This pattern of an initial NMDA-dependent fear conditioning yielding subsequent NMDA-independent fear conditioning is consistent with findings in adult animals, where animals show substantial fear to both conditioning contexts (Sanders & Fanselow, 2003; Tayler et al., 2011). This view suggests that while infant fear memories remain intact, interference in the behavioral expression of those memories exists.

Such interference in the behavioral expression of infant fear memories is consistent with the present data as well as previous studies showing that a reminder of the infant fear experience can “recover” the forgotten memory (e.g., Campbell & Jaynes, 1966; Davis and Rovee-Collier, 1983; Spear & Parsons, 1976). Once behavioral indices of forgetting have occurred, administration of a pharmacological agent such as epinephrine or FG-7142 (Haroutunian & Campbell, 1977; Kim et al., 2006; Tang, McNally, & Richardson, 2007) permits expression of the infant fear memory once again. In the present experiments, we see that rats exposed to 15 footshocks on PND17 show substantial fear of the context when tested 3 days later. Over time, this infant fear memory is gradually forgotten. On PND26, rats freeze in Context A approximately half as much as they did on PND20 and by PND92 they show no behavioral evidence of the memory at all. However, if rats receive a single “reminder” footshock in a novel context (Context B) on PND24, then freezing to the original context (Context A) is returned to its initial level when tested 2 days later. However, this was not the case for rats that received the reminder footshock on PND90, where the reminder had no impact on expression of fear memory for the original context. Taken together, these data suggest that a reminder of the original event can enhance subsequent retrieval of a fear memory that was acquired during infancy, as long as the reminder occurs at a time when there is still observable retrieval of the original event. Perhaps the reminder on PND24 allows for the retrieval of the original PND17 memory resulting in a strengthening of the original memory through reconsolidation processes. However, when the reminder is given on PND90, when there is no observable retrieval of the original PND17 memory, the memory is unable to be strengthened through reconsolidation and, thus, we see no increase in freezing as a result of the reminder. This is consistent with other studies showing a decrease in the ability for memories to become reconsolidated as they decay across time (e.g., Forcato, Fernandez, & Pedreira, 2013).

These findings suggest that early adverse experience can have persistent, possibly lifelong, consequences for learning about future threat. It is interesting that previous studies have found persistent deficits in adult fear learning as a result of early-life stress exposure during PND8–12 (Moriceau, Raineki, et al., 2009; Sevelinges et al., 2007). Thus, this stress experience occurs during the “stress hyporesponsive period”—a 2-week window at the beginning of life that is marked by blunted neuroendocrine response to stress (Sapolsky & Meaney, 1986; Schapiro, 1962). During this period, amygdala plasticity and fear responding are significantly suppressed (Moriceau & Sullivan, 2004). After this period, stress-induced corticosterone release permits amygdala activation and facilitates fear learning (Moriceau, Shionoya, et al., 2009). This suggests that stress exposures following the hyporesponsive period may lead to corticosterone-mediated enhancement of fear learning in the present study, via persistent amygdala activation (Kim et al., 2012). Taken together, these findings suggest that the developmental timing of early-life stress experiences may determine whether the resulting neuroadaptive changes will yield resiliency or vulnerability in the face of future adversity (Macrì, Zoratto, & Laviola, 2011).

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