

Juvenile female rats, but not male rats, show renewal, reinstatement, and spontaneous recovery following extinction of conditioned fear

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Anxiety disorders emerge early, and girls are significantly more likely to develop anxiety compared to boys. However, sex differences in fear during development are poorly understood. Therefore, we investigated juvenile male and female rats in the relapse behaviors following extinction of conditioned fear. In all experiments, 18-d-old rats first received three white-noise–footshock pairings on day 1. On day 2, extinction involved 60 white-noise alone trials. In experiment 1, we examined renewal by testing the rats in either the same or different context as extinction on day 3. Male rats did not show renewal, however, female rats showed renewal. Experiment 2 investigated reinstatement by giving rats either a mild reminder footshock or context exposure on day 3. When tested the next day, male rats did not show reinstatement, whereas female rats showed reinstatement. Experiment 3 investigated spontaneous recovery by testing the rats either 1 or 5 d following extinction. Male rats did not show any spontaneous recovery whereas female rats did. Taken together, fear regulation appear to be different in males versus females from early in development, which may explain why girls are more prone to suffer from anxiety disorders compared to boys.

Anxiety disorders are developmental disorders (Kessler et al. 2005; Merikangas et al. 2010) that occur more commonly in women (37.3%) compared to men (25.6%) (Kessler et al. 2012). Interestingly, studies show that individual differences in how we process fear emerge as early as 21 months of age with ~15% of children developing more intense anxiety-like temperament compared to their peers (Reznick et al. 1986; Kagan et al. 1988; Costello et al. 2005). This suggests that sex differences in anxiety disorders may also emerge early in life. Indeed, Lewinsohn et al. (1998) reported that by 6 years of age, girls are twice as likely to have experienced a clinically diagnosed anxiety disorder as boys.

Due to the presence of early sex differences in anxiety disorders, we explored fear learning in juvenile male and female rats. Pavlovian fear conditioning in developing rodents is widely used to better understand the fear learning that may occur in anxiety disorders, due to its rapid acquisition even in very young rodents (Kim and Richardson 2007a; Ganella and Kim 2014). It involves pairing an initially neutral conditioned stimulus (CS) (e.g., white noise) with an aversive, unconditioned stimulus (US) (e.g., electric footshock) that later allows the CS to elicit physiological fear responses without the US. This is referred to as a conditioned response (CR; e.g., freezing in rodents). Subsequently, the CR can be extinguished by repeated exposure to the CS without the US. This process is referred to as “extinction” and forms the basis of exposure therapies in the clinic (Maren et al. 2013).

It is widely accepted that extinction during adulthood forms a new memory of the CS-no US association, which competes with the previously acquired CS-US association memory (Bouton 2002). This is because the original CS-US fear memory can return following extinction. That is, expression of the extinction memory is intimately bound to the physical, internal, and temporal con-

texts in which extinction was learned (Bouton 2004). There are three major fear relapse behaviors that demonstrate the return of fear. Renewal describes the return of fear in a physical context other than where extinction occurred (Bouton and Bolles 1979). Reinstatement is the process in which extinguished fear may return if the subject encounters a reminder stimulus (Rescorla and Heth 1975). This can also be described as re-experiencing the fearful “internal context” of the original conditioning episode. Last, spontaneous recovery describes the return of extinguished fear after a temporal delay from extinction training, in which the longer the extinction-test interval, the more likely the conditioned fear memory will be expressed (Quirk 2002). These fear relapse behaviors are commonly observed in adult rodents (Burgos-Robles et al. 2007; Karpova et al. 2011) and humans (Dirikx et al. 2007, 2009; Huff et al. 2009; Schiller et al. 2010).

During the juvenile period (hereby defined as postnatal days (P) 14–21, (Madsen and Kim 2016), however, extinction may “erase” the original CS-US memory and relapse is not observed. We demonstrated that juvenile rats do not display renewal (Kim and Richardson 2007a) and reinstatement (Kim and Richardson 2007b). Furthermore, Gogolla et al. (2009) observed the absence of spontaneous recovery in juvenile mice. Together, these findings suggest that extinction erases conditioned fear memory in juvenile rodents. However, more recent findings are contradictory to these results. Specifically, Arias and his colleagues demonstrated that juvenile rats can in fact demonstrate renewal (Revillo et al. 2014a), reinstatement (Revillo et al. 2016), and spontaneous recovery

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(Revillo et al. 2014b), indicating that extinction may also be a new competing memory in juvenile rats.

A notable difference between these contradictory findings in juvenile rodents is that in most studies that observed no relapse following extinction exclusively used male rodents (Kim and Richardson 2007a,b; Gogolla et al. 2009), whereas studies that used either female rats or both sexes observed relapse following extinction. That is, only female juvenile rats were tested when renewal was observed (Revillo et al. 2013) and female and male juvenile rats were pooled across sexes when reinstatement and spontaneous recovery were observed (Revillo et al. 2014b, 2016). In light of observed sex differences in the prevalence of anxiety disorders during childhood, we hypothesized that the contradictory findings in extinction in juvenile rodents may be explained by differences in relapse following extinction between males and females even before puberty. Thus, we investigated renewal, reinstatement, and spontaneous recovery following extinction of conditioned fear in juvenile male and female rats in the present study.

Results

Renewal is not observed in juvenile male rats but it is observed in juvenile female rats

See Table 1 for the information on baseline levels of freezing and *n* for each behavioral phase in all experiments. P18 male rats acquired conditioned fear (Fig. 1A). Repeated-measures (RM) ANOVA yielded a significant main effect of Conditioning trial ($F_{(2,40)} = 19.630, P < 0.001$), but no effects of Condition and no interaction ($P_s > 0.05$) (Fig. 1A). The learned fear was extinguished during extinction in ABB and ABA groups, while the No-Extinction group displayed minimal freezing throughout the session (Fig. 1B). RM ANOVA yielded a significant main effect of Extinction block ($F_{(5,100)} = 22.385, P < 0.001$), Condition ($F_{(2,20)} = 5.434, P < 0.05$) and Extinction block \times Condition interaction ($F_{(10,100)} = 6.151, P < 0.001$). Post-hoc Tukey's honest significant difference (HSD) multiple comparison tests revealed that the rats in the No-Extinction group showed significantly less freezing than the ABB and ABA groups ($P_s < 0.05$). No other effects were observed ($P_s > 0.05$). At test, juvenile male rats did not show renewal (Fig. 1C). One-way ANOVA revealed a significant main effect of Condition ($F_{(2,20)} = 7.062, P < 0.01$). Post-hoc Tukey's HSD tests revealed that rats in the No-Extinction group demonstrated significantly higher freez-

ing than rats in the ABB and ABA groups ($P_s < 0.05$). No other effects were observed ($P_s > 0.05$).

P18 female rats acquired conditioned fear (Fig. 1D). RM ANOVA yielded a significant main effect of Conditioning trial ($F_{(2,68)} = 22.271, P < 0.001$), but no effects of Condition and no interaction ($P_s > 0.05$). The learned fear was extinguished in ABB and ABA groups during extinction, while the No-Extinction group showed minimal freezing (Fig. 1E). RM ANOVA yielded a significant main effect of Extinction block ($F_{(5,170)} = 40.269, P < 0.001$), Condition ($F_{(2,34)} = 9.769, P < 0.001$) and Extinction block \times Condition interaction ($F_{(10,170)} = 13.827, P < 0.001$). Post-hoc Tukey's HSD tests revealed that the No-Extinction group displayed significantly lower freezing than the ABB and ABA groups ($P_s < 0.05$). At test, P18 female rats displayed renewal (Fig. 1F). One-way ANOVA yielded a significant main effect of Condition ($F_{(2,34)} = 13.726, P < 0.001$) and post-hoc Tukey's HSD tests revealed that the ABA group displayed significantly higher freezing than the ABB group ($P < 0.05$). Furthermore, the No-Extinction group displayed significantly higher freezing than the other two groups ($P_s < 0.05$), indicating that while renewal occurred, it was not at the level of no extinction group.

Reinstatement is not observed in juvenile male rats, but it is observed in juvenile female rats

P18 male rats acquired conditioned fear (Fig. 2A). RM ANOVA yielded a significant main effect of Conditioning trial ($F_{(2,28)} = 3.709, P < 0.05$), but no effect of Condition and no interaction ($P_s > 0.05$). Extinction was also comparable across groups (Fig. 2B). RM ANOVA yielded a significant main effect of Extinction block ($F_{(5,70)} = 36.877, P < 0.001$) but no effect of Condition and no interaction ($P_s > 0.05$). At test, juvenile male rats did not demonstrate reinstatement (Fig. 2C). An independent groups *t*-test yielded no significant effect ($P > 0.05$).

P18 female rats acquired conditioned fear (Fig. 2D). RM ANOVA yielded a significant main effect of Conditioning trial ($F_{(2,26)} = 4.019, P < 0.05$), but no effects of Condition and no interaction ($P_s > 0.05$). Extinction was also comparable across groups (Fig. 2E). RM ANOVA yielded a significant main effect of Extinction block ($F_{(5,65)} = 18.360, P < 0.001$) but no effect in Condition and no interaction ($P_s > 0.05$). At test, the female rats that received a reminder foot-shock displayed significantly higher freezing compared to the rats that did not receive the reminder foot-shock. An independent groups *t*-test yielded a significant

Table 1. Mean \pm SEM Baseline freezing levels and *n* per group for all experiments

Experiment	Group	Conditioning	Extinction	Test	<i>N</i>
1A	No-Extinction	1.89 \pm 0.70	23.00 \pm 5.88	4.10 \pm 3.48	<i>n</i> = 8
	ABB	2.43 \pm 1.57	14.04 \pm 5.93	8.56 \pm 5.53	<i>n</i> = 8
	ABA	3.50 \pm 2.90	21.12 \pm 14.40	15.58 \pm 11.81	<i>n</i> = 7
1B	No-Extinction	2.17 \pm 1.26	13.68 \pm 5.83	7.67 \pm 4.96	<i>n</i> = 12
	ABB	0.88 \pm 0.48	9.24 \pm 3.87	2.93 \pm 1.54	<i>n</i> = 11
	ABA	2.45 \pm 0.89	26.37 \pm 8.47	19.67 \pm 8.01	<i>n</i> = 14
2A	No-Reminder	2.51 \pm 0.87	20.58 \pm 6.42	6.29 \pm 2.87	<i>n</i> = 8
	Reminder	1.21 \pm 0.53	29.16 \pm 7.58	31.34 \pm 11.48 ^a	<i>n</i> = 8
2B	No-Reminder	0.63 \pm 0.34	16.34 \pm 3.09	15.15 \pm 7.37	<i>n</i> = 8
	Reminder	0.74 \pm 0.40	19.14 \pm 7.87	26.87 \pm 5.70	<i>n</i> = 7
3A	Day 1	2.61 \pm 1.41	27.79 \pm 8.87 ^a	2.72 \pm 1.23	<i>n</i> = 11
	Day 5	1.38 \pm 0.46	8.15 \pm 3.27	10.29 \pm 9.41	<i>n</i> = 11
3B	Day 1	2.22 \pm 0.71	17.41 \pm 7.55	1.96 \pm 1.55	<i>n</i> = 9
	Day 5	6.77 \pm 2.85	22.84 \pm 6.14	8.97 \pm 5.78	<i>n</i> = 9

^aSignificant main effect of condition ($P_s < 0.05$). For sessions where there was a significant difference in baseline freezing, the CS-elicited freezing data were also analyzed using an analysis of covariance (ANCOVA) with baseline freezing as a covariate, as previously described (Kim and Richardson 2007a). ANCOVA revealed that the baseline levels of freezing did not predict CS-elicited freezing at any session ($P_s > 0.05$). Furthermore, the statistical outcome was the same for ANOVA/*t*-test or ANCOVA, therefore we reported ANOVA/*t*-test throughout this manuscript.

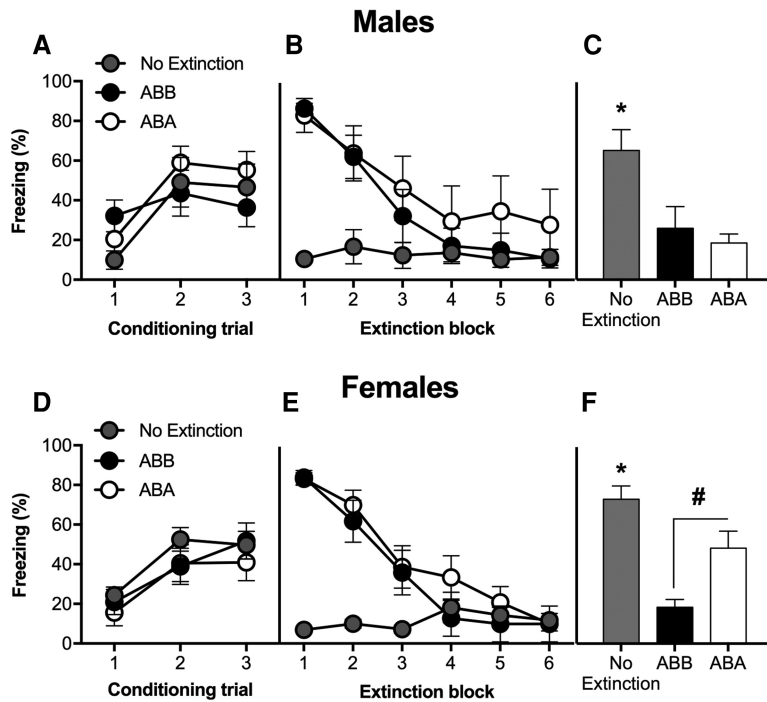


Figure 1. Mean (\pm SEM) CS-elicited freezing at conditioning, extinction and test in Experiments 1A and 1B. (A,D) Rats showed comparable CS-elicited freezing during conditioning. (B,E) During extinction, the ABB and ABA groups showed comparable extinction, while No-Extinction group displayed minimal freezing. (C) P18 male rats did not display renewal. (F) P18 female rats showed renewal. (*) $P < 0.05$ No-extinction group significantly froze more than other groups at test. # $P < 0.05$ ABA group froze more than ABB group at test.

main effect of Condition ($t_{(13)} = 9.974$, $P < 0.01$). That is, P18 female rats demonstrated reinstatement (Fig. 2F).

Spontaneous recovery is not observed in juvenile male rats but it is observed in juvenile female rats

P18 male rats acquired conditioned fear (Fig. 3A). RM ANOVA yielded a significant main effect of Conditioning trial ($F_{(2,40)} = 7.166$, $P < 0.01$), but no effects in Test Day and no interaction ($P_s > 0.05$). Extinction was also comparable across groups (Fig. 3B). RM ANOVA yielded a significant main effect of Extinction block ($F_{(5,100)} = 21.072$, $P < 0.001$), but no effect of Test Day and no interaction ($P_s > 0.05$). At test, P18 male rats did not demonstrate spontaneous recovery (Fig. 3C). An independent groups t -test yielded no effect ($P > 0.05$).

P18 female rats acquired conditioned fear (Fig. 3D) RM ANOVA yielded a significant main effect of Conditioning trial ($F_{(2,32)} = 6.083$, $P < 0.01$) but no effect of Test Day and no interaction ($P_s > 0.05$). Extinction was also comparable across groups (Fig. 3E). RM ANOVA yielded a significant main effect of Extinction blocks

($F_{(5,80)} = 22.173$, $P < 0.001$) and no effect of Test Day and no interaction ($P_s > 0.05$). At test, P18 female rats displayed spontaneous recovery (Fig. 3F). That is, rats tested 5 d after extinction displayed significantly higher freezing compared to rats tested the day after extinction ($t_{(16)} = 5.270$, $P < 0.05$).

Discussion

The present study demonstrates that P18 female rats showed renewal, reinstatement and spontaneous recovery while P18 male rats did not display any of these behaviors. Importantly, there was no sex effects during conditioning and extinction across all experiments (smallest $P = 0.1$), indicating that the presence of relapse effects in females is not due to stronger fear conditioning or weaker extinction compared to males in our study. The absence of the fear relapse behaviors in juvenile male rats is consistent with previous studies (Gogolla et al. 2009; Kim and Richardson 2007a,b) that directly compared P17 and P23 male rodents and observed that P23 male rodents showed relapse following extinction whereas P17 male rodents did not. Taken together, it appears that extinction learning erases conditioned fear

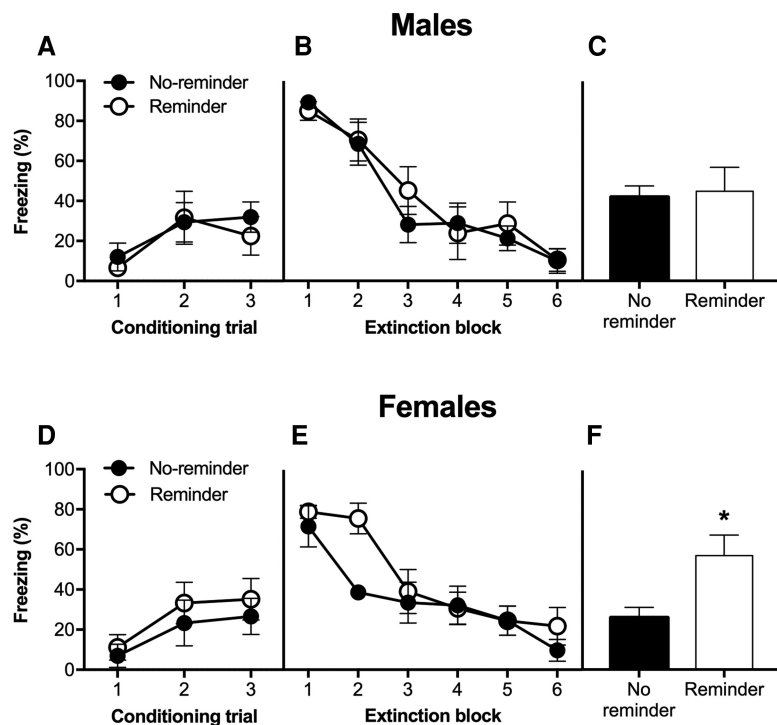


Figure 2. Mean (\pm SEM) CS-elicited freezing at conditioning, extinction and test in Experiments 2A and 2B. All rats showed comparable CS-elicited freezing during (A,D) conditioning and (B,E) extinction. (C) At test, P18 male rats did not display reinstatement, whereas (F) P18 female rats did; (*) $P < 0.05$ reminder versus no-reminder group at test.

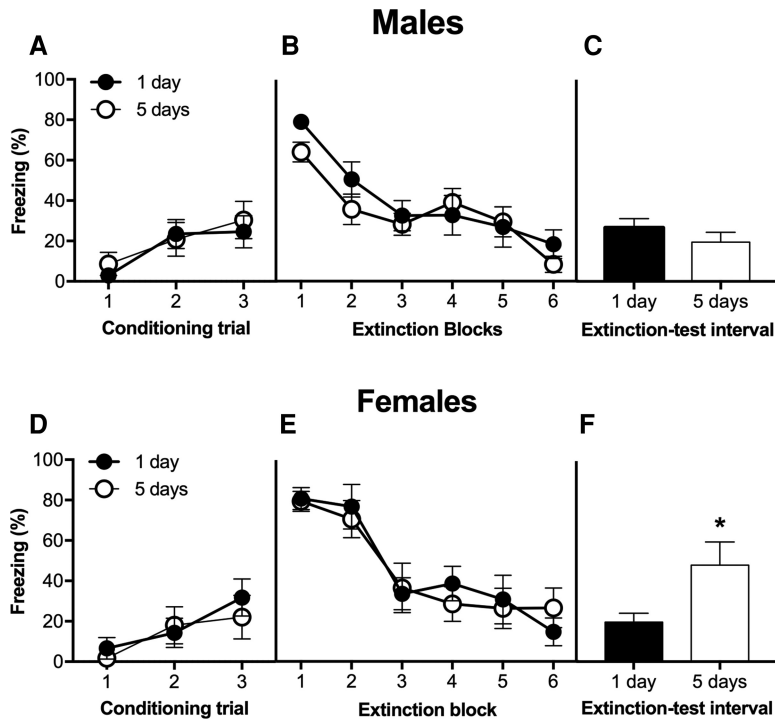


Figure 3. Mean (\pm SEM) CS-elicited freezing at conditioning, extinction and spontaneous recovery test in Experiments 3A and 3B. Rats showed comparable CS-elicited freezing during (A, D) conditioning and (B, E) extinction. (C) At test, P18 male rats did not display spontaneous recovery, whereas (F) P18 female rats did. (*) $P < 0.05$ rats that were tested after 5 d following extinction froze more than rats tested after 1 d.

memory in P17 male rats. This is supported by the previous pharmacological and intracranial studies showing a dissociated neural circuitry of fear extinction across development in male rodents (Kim and Richardson 2007b, 2008, 2009, 2010a; Gogolla et al. 2009; Ganella et al. 2016). Notably, there have been studies that reported reinstatement and spontaneous recovery following fear extinction in juvenile rats (Revilla et al. 2014b, 2016). While those studies explicitly analysed males and females and reported that sex did not interact with group conditions, the main effects for sex were not described. In addition, statistical power may have been too low to detect any sex effects with pooled n 's of 8–11 per group, which would leave 4–6 rats per sex.

We observed relapse following fear extinction in juvenile female rats in the present study, which is consistent with renewal of fear in P17 female rats previously reported (Revilla et al. 2014a), as well as with findings in adult female rodents (Baran et al. 2009; Hoffman et al. 2010; Baker-Andresen et al. 2013; Fenton et al. 2014). This suggests extinction learning in P18 female rats may be sufficiently mature to express adult-like extinction learning, in which a new safety memory is formed to inhibit the conditioned fear memory. Additionally, P18 female rats may learn extinction in a context-dependent manner while P18 male rats learn extinction in context-independent manner. Such extinction differences so early in life may indicate that juvenile male rats are impaired in contextual learning compared to juvenile female rats. However, we recently addressed whether the failure to observe renewal in P18 male rats is in fact due to their deficits in contextual conditioning. To our surprise, both P18 and P25 male rats showed comparable contextual fear learning at both the immediate and 24-h delayed tests following conditioning, whereas renewal was only observed in P25 rats and not in P18 rats (Park et al. 2017). That is, context-dependent fear extinction and contextual fear conditioning mechanisms are dissociated in P18 rats.

Furthermore, the majority of previous studies that investigated contextual fear learning in juvenile rodents pooled males and females (Rudy 1993; Rudy and Morledge 1994; McKinzie and Spear 1995; Brassler and Spear 1998; Esmoris-Arranz et al. 2008), which makes it difficult to ascertain whether female rats can learn contextual fear. Indeed, we recently observed that P18 female rats display a deficit in contextual fear learning compared to P25 female rats (CHJ Park, DE Ganella, JH Kim, unpublished observations). In fact, the present results support differences in contextual fear learning between male and female juvenile rats. In experiment 2A, male rats that received the reminder US displayed significantly higher freezing during the baseline period at test compared to male rats that did not receive any reminder US. Reminder treatment and the test occurred in the same context (Table 1). This suggests that the reminder session may have caused contextual fear learning in P18 male rats. In contrast, the reminder US did not cause a significant elevation in baseline freezing at test in juvenile female rats in experiment 2B. Importantly, relapse of extinguished fear appears not to be driven by baseline levels of freezing in the present study. Specifically, the relapse condition show higher levels of baseline freezing levels at test in all experiments in both males and females (although only experiment 2A was significant). For example, in experiments 1A and 1B, the ABA groups at test seemed to display higher levels of baseline freezing compared to the other groups. However, only females showed relapse at test and males did not. This dissociation between freezing during the baseline period versus the CS is consistent with previous studies that show baseline levels of freezing do not predict freezing to the CS at test (Kim and Richardson 2007a,b).

Taken together, differences in relapse of extinguished fear between males and females observed in the present study appear to be dissociated with contextual fear conditioning abilities in juvenile rats. The hippocampus is crucially involved in both learning mechanisms in adult rodents (Phillips and LeDoux 1992; Maren 2011). However, the hippocampus is anatomically divided into the dorsal (DH) and ventral hippocampus (VH), and these subregions are suggested to serve different functions (see Fanselow and Dong 2010, for review). In support of this idea, there is more evidence for the specific involvement of the DH in context fear learning (Kim and Fanselow 1992; Hunsaker and Kesner 2008) while the VH may specialize in context-specific extinction learning (Hobin et al 2006; Adhikari et al 2010; Orsini et al 2011). It should be noted there are also conflicting results in the role of DH versus VH. For example, Corcoran et al. (2005) demonstrated that DH inactivation impaired the expression of context-specific extinction learning. Zhang et al. (2014) also showed that VH or DH inactivation had a similar outcome of disrupting contextual fear at test when infused before conditioning. Future studies need to determine any overlaps and dissociations in function of VH and DH in learned fear behaviors by targeting more specific subregions.

Considering that the hippocampus undergoes rapid changes in developing rodents (Semple et al. 2013), it may be the case

that the sequence of the hippocampal maturation is differentiated in males and females. The DH in P18 male rats may be mature while the VH remains immature as we observed that P18 male rats showed contextual fear learning but not context-dependent extinction (Park et al. 2017). Conversely, P18 female rats may have a deficit in contextual fear learning but show context-specific extinction, suggesting that their VH matures before DH. Alternatively, males and females may engage in DH and VH differently in general. For example, Keiser et al. (2017) investigated sex differences in contextual fear generalization and the involvement of the DH in adult mice. They reported that the CA1, CA3, and DG regions of the DH were activated in the males, whereas the females engaged the CA1 region of the DH following contextual fear learning (Keiser et al. 2017). Future studies should examine whether VH is also engaged differently in males and females after contextual fear learning in both juveniles and adults.

The present findings open up many questions as to whether other characteristics of fear extinction in male and female juvenile rats are similar. For example, male juvenile rats show extinction independent of the medial prefrontal cortex (Kim et al. 2009) and N-methyl-D-aspartate receptor signaling (Langton et al. 2007; Kim and Richardson 2010b). Furthermore, extinction retrieval was not impaired with a pretest injection of the gamma-aminobutyric acid inverse agonist FG7142 (Kim and Richardson 2007a). On the other hand, extinction in juvenile male rats does require the amygdala (Kim and Richardson 2008), endogenous opioids signaling (Kim and Richardson 2009), and metabotropic-glutamate receptor 5 signaling (Ganella et al. 2016). It would be interesting to test those features of extinction in male and female juvenile rats.

It should be noted that our results are also consistent with the observation of renewal, reinstatement and spontaneous recovery following extinction of conditioned taste aversion in juvenile female rats (Revillo et al. 2014a). It is unknown whether relapse following extinction of conditioned taste aversion can also be observed in juvenile male rats. Conditioned taste aversion learning emerges earlier than conditioned freezing in rats (Hunt and Campbell 1997). For example, P14-15 rats acquired conditioned taste aversion in Revillo et al., (2014a), whereas fear extinction studies typically use P16 or older rats (Kim and Richardson 2010a). It may be the case that the neural mechanisms underlying extinction of conditioned taste aversion versus conditioned fear overlap. If that is the case, we expect failure of relapse behaviors following extinction of conditioned taste aversion in P14 male rats.

Taken together, our findings add to the growing literature on the prepubertal ontogeny of sexually dimorphic behaviors. For example, P21 male rats showed faster acquisition in spatial learning compared to P21 female rats in the Morris Water Maze (Cimadevilla et al. 1999). Recently chronic corticosterone treatment in adult male mice was shown to delay fear extinction in P14 female but not in P14 male offspring, while trait anxiety is increased in those male but not in female offspring (Short et al. 2016). It was also shown in mice that exercising fathers can reduce trait anxiety as tested by light-dark box tests in male but not female offspring aged P14 (Short et al. 2017). Notably, Short et al. (2017) demonstrated exercise changed expression levels of 84 microRNAs in the sperm of paternal breeders compared to the controls that may have subsequently changed in fear and anxiety behaviors differently in the male compared to female offspring. This finding suggests a genetic basis for sexually dimorphic behaviors before puberty. Indeed, Wijchers and Festenstein (2011) suggested sex chromosomes epigenetically modify gene expressions and contribute to sexually dimorphic brain formation and behaviors from very early in life. Recent findings further support this idea. At as early as 1 d of age, female rodents displayed a much higher DNA methylation at CpG sites brain-wide, and higher concentrations of certain DNA modifying enzymes (e.g., DNMT3a) in

the amygdala, compared to males (Kolodkin and Auger 2011; Nugent et al. 2015). Future studies should examine the contribution of such epigenetic differences between males and females in the ontogeny of sexually dimorphic behaviors, which will enormously facilitate our understanding why boys and girls are different in various facets of mental disorders.

In conclusion, the present study shows that prepubertal female rats show relapse following fear extinction but males do not. These findings are novel and explain inconsistencies in the literature. Furthermore, these behavioral findings in juvenile female rats provide an ethological relevant model of prevalence of anxiety disorders shown by female children, so that future studies can investigate facilitating extinction and reducing relapse in this vulnerable group.

Materials and Methods

Subjects

Experimentally naïve male and female Sprague-Dawley rats that were drawn from 33 different litters were used for the present study (bred and born in the colony at the Florey Institute of Neuroscience and Mental Health). The rats were P18 (± 1) at the start of behavioral experimentation. All rats were housed with their littermates and dam in individually ventilated-cages, under a 12 h/12 h cycle (lights on at 7:00am). Food and water were available ad libitum. No more than one rat per sex per litter per group was used. In every litter, both male and female rats were used to run the experiments concurrently (e.g., experiment 1A and 1B were run concurrently, sometimes even with experiments 2A and 2B), although the numbers of females and males drawn per litter were not always matched, depending on pup availability. All rats were treated in accordance with *The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (NHMRC 2004) and all procedures were approved by the Animal Care and Ethics Committee at the *Florey Institute of Neuroscience and Mental Health*.

Apparatus

All chambers were equipped with a Med Associates *VideoFreeze* System (Med Associates, VT, USA) and enclosed in a sound attenuating box with a near infrared (NIR) light source as described previously (Park et al. 2017). Two distinct contexts/chambers were housed in separate rooms and were arbitrarily named Context A and B, and they differed in shape, visual, and olfactory cues as previously described (Park et al. 2017).

Procedures

Experiment 1

On day 1, rats received fear conditioning, which involved a 2-min baseline period followed by three CS-US pairings in Context A. The pairings involved a 10 sec CS (white noise, 67 dB) that coterminated with a 1s US (foot-shock, 0.6 mA) and the mean inter-trial interval (ITI) was 110 sec. On day 2, two thirds of the rats received extinction training in Context B (ABA and ABB groups). This involved a 2-min baseline period followed by 60 trials of 10 sec CS and the ITI was 10 sec. The remaining rats received “No-extinction”, which involved an exposure to Context B without the CS trials over an equivalent time as the extinction groups. On day 3, the rats were tested in either Context A or B. This involved a 1-min baseline period followed by a 2-min CS. There were no differences in the levels of freezing in the rats that were tested in context A or B for No extinction group ($P > 0.05$), so these rats were pooled into the group “No extinction.”

Experiment 2

The behavioral procedures for conditioning and extinction were the same as described in experiment 1, except “No extinction” group was not included. On day 3, a half of the rats received

a mild reminder US (foot-shock, 1 sec, 0.3 mA) in Context B after a 2-min baseline period. The other half of the rats underwent “No-reinstatement” procedure that involved exposure to Context B for the same amount of time, without the reminder US. On day 4, the rats were tested in Context B as described in experiment 1.

Experiment 3

The behavioral procedures for conditioning, extinction, and test were the same as described in experiment 1, except that rats were tested either 1 or 5 d after extinction.

Scoring and statistical analyses

Freezing was calculated via automated near-infrared video tracking equipment and computer software (*VideoFreeze*, Med Associates, VT, USA), as previously described (Ganella et al. 2016). A trained scorer blinded from experimental groups manually scored 25% of all rats in a time-sampling manner. Afterwards, the motion threshold with the highest correlation with the manual scoring (threshold 70 and freezing duration 30 frames) was chosen for the VideoFreeze automated calculation of freezing. This is consistent with a previous study using P17 and P24 rats (Ganella et al. 2016).

In all experiments, 60 CS trials during extinction training were averaged to 6 extinction blocks, where each block was an average of 10 CS trials. The data were analyzed by analysis of variance (ANOVA), analysis of covariance (ANCOVA), or t-tests using SPSS (IBM Corp., New York, USA).

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