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Brief report

Context fear learning and renewal of extinguished fear are dissociated in juvenile female rats.

Running title: Contextual memory in juvenile female rats

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract

Extinction is the decrease in emotion to a cue that was previously associated with an emotionally significant event. It involves repeated presentation of the cue without any consequences. In adult animals, extinguished fear to a cue can return if the cue is presented in a different environment/context to where extinction occurred, referred to as renewal. We have previously reported that developing female, but not male, rats show renewal. This study investigates whether the ability of developing female rats to show renewal is related to their ability in fear conditioning to the context. Additionally, facilitation of context conditioning by weaning previously shown in male rats was tested in developing female rats. In experiment 1, postnatal day 25 (P25) and P18 females rats showed renewal. P25 rats show more fear overall, suggesting a weaker extinction recall in this age. Experiment 2 tested context- and cue-elicited fear either immediately or 24 hours following conditioning. At the immediate test, P18 rats showed less context-fear compared to P25 rats. All rats showed low levels of context-fear at the 24 hours test. There were no age differences in cued fear. Weaning at P21 did not affect context or cue memory in P25 female rats. These findings suggest that the ability to form contextual fear memory is unrelated to the expression of renewal in juvenile female rats.

Keywords: Extinction, context, fear, renewal, development, female, weaning

INTRODUCTION

Anxiety disorders are developmental disorders that are more prevalent in females than males (Kessler et al., 2012; McLean et al., 2011) and this sex difference is observed as early as six years of age (Anderson et al., 1987; Lewinsohn et al., 1998). However, the development of fear regulation in females is poorly understood compared to fear regulation in males (Donner & Lowry, 2013; Lebron-Milad & Milad, 2012). Pavlovian fear conditioning is widely used to study fear regulation. In this paradigm, a neutral conditioned stimulus (CS) (e.g., white-noise) is repeatedly paired with an aversive unconditioned stimulus (US) (e.g., footshock). As a result, the CS alone can elicit fear. This fear can then be extinguished by repeatedly presenting the CS without the US.

In adults, it is widely accepted that this extinction process forms a new memory of the CS-no US association that competes with the previously acquired CS-US association (Bouton, 2004; Karpova et al., 2011; Schiller et al., 2008). This is because extinction expression is dependent on the surrounding environment, and the original fear can return when the extinguished cue is tested in a different context to where extinction occurred. Such return of extinguished fear is referred to as renewal. However, extinction is different in developing rodents. Specifically, extinguished fear has been shown to relapse in adult rodents but not in rodents aged postnatal day (P) 15–19 (Kim & Richardson, 2007; Schiller et al.,

2008). This has led to the idea that extinction can permanently reduce conditioned fear memory early in life (Ganella & Kim, 2014; Kim, 2017; Maren, 2011).

Interestingly, we recently observed renewal of extinguished fear in P18 female rats (Park, Ganella, & Kim, 2017b). Considering the importance of the context in the return of extinguished fear (Bouton, 2004), these findings suggest that female juvenile rats can form contextual memories while male juvenile rats cannot. However, contextual learning and context-dependent extinction are dissociated, at least in juvenile male rats. Specifically, P18 and P25 male rats showed comparable immediate and long-term contextual fear memory, despite showing differences in renewal (Park, Ganella, & Kim, 2017a).

No one has yet explicitly compared renewal and contextual memory in developing female rats. With emerging evidence of prepubertal sex differences in learning and memory (Cimadevilla et al., 1999; Park, Ganella, & Kim, 2017b; Short et al., 2016, 2017), this is a significant gap in the literature that needs to be addressed for us to further understand why anxiety disorders are neurodevelopmental disorders. Therefore, we investigated renewal of extinguished fear and contextual fear learning in juvenile (i.e. P18) and preadolescent (i.e. P25) female rats. Additionally, we examined the effect of weaning at P21 on contextual fear memory in P25 female rats based on our previous finding that weaning rats at P21 significantly increases contextual fear learning in P25 male rats (Park, Ganella, & Kim, 2017a). Given that the expression of renewal and contextual fear are dissociated in developing male rats (Park, Ganella, & Kim, 2017b), we expected that they may also be dissociated in developing female rats, with weaning facilitating contextual fear expression.

METHODS

Subjects

A total of 133 female Sprague-Dawley rats across 47 litters were used (in-house colony at the Florey Institute of Neuroscience and Mental Health). Rats were either P18 (± 1) or P25 (± 1) at the start of the behavioural experimentation. All rats were housed with their littermates together with the dam in individually ventilated cages under a 12h/12h cycle (lights on at 6:00am), except in experiment 2, where a group of rats were weaned at P21 as reported in a previous study (Park et al., 2017a). Food and water were available *ad libitum*. No more than one rat per litter was used per group. All rats were treated in accordance with *The Australian*

1 *Code of Practice for the Care and Use of Animals for Scientific Purposes* (NHMRC, 2004)
2 and all procedures were approved by the Animal Care and Ethics Committee at the Florey
3 Institute of Neuroscience and Mental Health.

4 *Apparatus*

5 Rectangular chambers (31.8 cm x 25.4 cm x 26.7 cm) with stainless steel rods were equipped
6 with a Med Associates *VideoFreeze* System (Med Associates, VT, USA) and enclosed in a
7 sound attenuating box with a near infrared (NIR) light source as previously described
8 (Ganella, Thangaraju, Lawrence, & Kim, 2016). Each chamber was equipped with a speaker
9 and a monochrome camera with a NIR-pass filter that video-recorded all experimental
10 sessions. Two distinctive contexts were housed in separate rooms and were arbitrarily named
11 Context A (white house-light, round stickers on the back wall, woodchip bedding beneath the
12 grid floor, eucalyptus-scented cleaning agent) and B (white stimulus-light, a curved wall,
13 paper towels beneath the grid floor, 80% ethanol cleaning agent).

14 *Procedures*

15 **Experiment 1.** Design was a 2×2 , with the first factor being Age (P18 vs P25) and the
16 second factor Test Context (Same vs Different to the extinction context). On day 1, after a 2-
17 min baseline period, rats received 3 CS-US pairings in Context A. Each CS-US pairing
18 involved 10s CS (white noise, 67dB) that co-terminated with the 1s US (footshock, 0.6 mA).
19 The mean inter-trial interval (ITI) was 110s. On day 2, after a 2-min baseline period, rats
20 received 60 trials of 10s CS with 10s ITIs in Context B. On day 3, rats were placed in either
21 Context A or Context B to test for renewal. After a 1-min baseline period, 2-min CS was
22 presented.

23 **Experiment 2.** Design was a 3×2 , with the first factor being Group (P18 unweaned vs P25
24 unweaned vs P25 weaned) and the second factor Test Interval (Immediate vs 24 hours). On
25 day 1, the rats were conditioned in Context A as described in experiment 1. Either as soon as
26 the chambers were cleaned (i.e. Immediate) or 24 hours from fear conditioning, the rats were
27 then returned to the conditioning chamber for 5 minutes to test context fear. Following
28 context test, rats were returned to their home cages for an hour and were then placed in
29 Context B for the cue test as described in experiment 1.

30 *Scoring and Statistical Analyses*

NIR video tracking and computer software automatically calculated freezing (motion threshold 70, freezing duration 30 frames, *VideoFreeze*, Med Associates, VT, USA) as previously described (Ganella et al., 2016). During conditioning, the first 9 seconds of the CS was analysed to avoid the movement induced by footshock, whilst extinction trials were divided into 6 blocks for analyses. Analysis of variance (ANOVA) was carried out on SPSS (IBM Corp., New York, USA). Significant interactions were followed up with *post hoc* t-tests in experiment 1 (due to 2 groups per factor), and Tukey's honest significant difference (HSD) *post hoc* tests in experiment 2. For sessions with significant baseline differences (Table 1), data were also analysed as an analysis of co-variance (ANCOVA) with the baseline as a covariate as previously described (Kim & Richardson, 2007). ANCOVA revealed that the baseline levels of freezing did not predict CS-elicited freezing at any session ($p > 0.05$). Furthermore, the statistical outcome was the same for ANOVA or ANCOVA, therefore we reported ANOVA throughout this manuscript.

RESULTS

Experiment 1: P18 and P25 female rats show renewal of conditioned fear

During conditioning, rats similarly increased freezing to the CS (Figure 1A). Repeated-measures (RM) ANOVA yielded a significant effect of Conditioning Trial ($F_{(2,102)} = 25.398$, $p < 0.0001$) but no other effects ($p > 0.05$). Similarly, extinction was not different between groups (Figure 1B). RM ANOVA yielded a significant effect of Extinction Block ($F_{(5,255)} = 87.362$, $p < 0.0001$) but no other effects ($p > 0.05$). At test, although both ages displayed renewal, P25 rats froze more compared to P18 rats (Figure 1C). Two-way ANOVA yielded a significant effect of Test Context ($F_{(1,51)} = 10.884$, $p < 0.05$) and Age ($F_{(1,51)} = 7.107$, $p < 0.05$). Test Context x Age interaction was not significant ($p > 0.05$).

Experiment 2: P18 female rats show impaired contextual fear compared to P25 female rats when tested immediately after fear conditioning.

Consistent with experiment 1, rats similarly increased freezing to the CS during conditioning (Figure 1D). RM ANOVA yielded a significant main effect of Conditioning Trial ($F_{(2,144)} = 59.365$, $p < 0.0001$) but no other effects ($p > 0.05$). P18 rats showed reduced contextual

freezing compared to P25 rats when tested immediately after conditioning (Figure 1E). Two-way ANOVA yielded a significant effects of Test Interval ($F_{(1,72)} = 23.546$, $p < 0.0001$), Group ($F_{(2,72)} = 10.545$, $p < 0.0001$), and a Group x Test Interval interaction ($F_{(2,72)} = 4.299$, $p < 0.05$). *Post-hoc* Tukey HSD tests between the rats tested at the immediate interval showed that P18 rats displayed significantly lower freezing to the context ($ps < 0.05$) compared to both P25 groups that did not differ ($p > 0.05$). No group differences were observed at the 24 hours test ($ps > 0.05$). At cue test, two-way ANOVA yielded a significant effect of Test Interval ($F_{(1,72)} = 19.893$, $p < 0.0001$) but no other effects ($ps > 0.05$), indicating that CS-elicited freezing increased between immediate to 24 hours test interval (Figure 1F).

DISCUSSION

The present study investigated the potential differences in contextual modulation of extinction and contextual learning between juvenile (P18) and preadolescent (P25) female rats. In experiment 1, both ages displayed renewal of extinguished fear, suggesting extinction was context-specific in these rats. Additionally, P25 rats displayed higher overall freezing at test compared to P18 rats regardless of the test context. In experiment 2, juvenile rats showed reduced contextual freezing compared with P25 rats, when tested immediately after conditioning. No age differences were observed for cue-elicited freezing. Lastly, weaning at P21 did not affect contextual or cue learning in P25 rats. Together, these findings indicate context-specific fear extinction and contextual fear learning are dissociated in developing female rats.

While renewal in P18 female rats was previously reported (Park et al., 2017b; Revillo, Molina, Paglini, & Arias, 2013) this study is the first to demonstrate renewal in P25 female rats. This indicates extinction is context-specific and therefore adult-like at least from P18 in female rats, which suggests that female rodents may mature faster than males that express renewal at P23 (Kim & Richardson, 2010). Interestingly, P25 female rats froze more than P18 rats at test, regardless of the context (Figure 1C). This effect of age at test was observed despite similar levels of freezing across the ages during conditioning and extinction, which highlights that P25 female rats are impaired in extinction recall compared to P18 female rats. This finding is reminiscent of our previous studies in adolescent male rats. Specifically, P35 rats show similar conditioning and extinction learning to P23 rats but are impaired in extinction recall (Kim, Li, & Richardson, 2011; McCallum, Kim, Richardson, 2010). Considering that puberty and related changes in the brain occur ~10 days earlier in females

1 than males (Willing & Juraska, 2015), these results strongly suggest that at least in rats,
2 females develop more rapidly than males in fear extinction.

3 Retention of contextual information is critical for renewal (Bouton, 2004) and
4 context-elicited freezing (Rozeske, Valerio, Chaudun, & Herry, 2015). Therefore, P18 female
5 rats displaying little evidence of context fear regardless of time of test, was unexpected. We
6 used parameters that facilitate contextual learning in juvenile rodents, such as distinct odours
7 (Brasser & Spear, 1998, 2004; Esmoris-Arranz, Mendez, Spear, Esmoris-Arranz, & Méndez,
8 2008; McKinzie & Spear, 1995) and presence of the CS during conditioning (Brasser & Spear,
9 2004). Identical parameters yielded 40-60% of freezing in P18 male rats even at 24 hours test
10 (Park, Ganella, & Kim, 2017a). Taken together, juvenile male but not female rats show
11 context conditioned fear.

12 Although males and females were not analysed separately, Travaglia et al. (2016)
13 demonstrated that P17 rats can exhibit inhibitory avoidance, a form of context memory. Its
14 long-term retention required the switch in N-methyl-D-aspartate (NMDA) receptor 2B
15 subunit-dominance to 2A subunit-dominance. Across weeks 3-4 of postnatal development,
16 NMDA receptor composition naturally shifts from 2B- to 2A-dominance in the hippocampus
17 (Kim, Perry, Ganella, & Madsen, 2017). Accelerating such switch via increases in brain-
18 derived neurotrophic factor or metabotropic glutamate receptor 5 signalling in the dorsal
19 hippocampus (dHPC) at P17 led to long-term retention of inhibitory avoidance memory,
20 suggesting that contextual representation is formed at P17 but its association with shock can
21 remain dormant without the 2B- to 2A switch (Travaglia et al., 2016).

22 While P25 females showed robust contextual freezing immediately after conditioning,
23 it was reduced to the level of P18 females the next day. This is inconsistent with a previous
24 finding that P24 female rats sustained robust freezing to the shocked context even 14 days
25 after conditioning (Colon, Odynocki, Santarelli, & Poulos, 2018). The shock was 1 mA in
26 that study and 0.6 mA in the present study, hence the discrepancy between the studies may be
27 quantitative. Nevertheless, observing renewal but not long-term context freezing using an
28 identical protocol in P17 and P25 female rats highlight that the expression of renewal is not
29 tied to the expression of context fear memory.

30 Weaning at P21 did not affect context freezing in P25 females, while it increased
31 context freezing in P25 male rats (Park, Ganella, & Kim, 2017a). In that study, we reasoned
32 that weaning at P21 is a stressor that increases blood corticosterone level that may impact the

1 hippocampal neurogenesis for males (Kikusui, Ichikawa, & Mori, 2009; Kikusui & Mori,
2 2009). The present findings are consistent with a study in adult mice showing that the
3 hippocampus is recruited in females significantly less than males during context fear retrieval
4 (Keiser et al., 2017). This suggests that whilst weaning may affect the hippocampus in both
5 sexes, context memory in females may be less affected by weaning due to the reduced
6 involvement of the hippocampus in context memory in females.

7 Together with the findings of Park et al. (2017a), our results strongly suggest that the
8 developmental emergence of context memory may be sex-dependent with context-specific
9 extinction learning emerging first in females while context fear learning emerging first in
10 males (Table 2). We speculate that such divergence is driven by different development of the
11 neural circuitry underlying fear learning and memory in males vs females. Context
12 conditioned fear and context-specific extinction critically involve the amygdala, medial
13 prefrontal cortex (mPFC), and dHPC/ventral hippocampus (vHPC) (Goode & Maren, 2018;
14 Rozeske et al., 2015). These regions have been largely identified by examining only males
15 (Lebron-Milad & Milad, 2012). In fact, these brain regions are sexually dimorphic due to a
16 high density of gonadal hormone receptors (Lebron-Milad & Milad, 2012). For example, the
17 mPFC is structurally asymmetric in P18 males but not in female rats (Van Eden, Uylings, &
18 Van Pelt, 1984) which suggests sex differences in neuronal population and organisation
19 (Willing & Juraska, 2015). Female rats show significant neuronal loss in the mPFC during
20 preadolescence due to gonadal hormones (Koss, Lloyd, Sadowski, Wise, & Juraska, 2015),
21 while this does not occur in male rats (Willing & Juraska, 2015). While sex differences in the
22 juvenile hippocampal structure are unknown, in preadolescent rodents males perform
23 significantly better than females in hippocampus-dependent tasks (Cimadevilla et al., 1999;
24 Roof, 1993b). Amygdala development is also sexually dimorphic, with medial amygdala
25 volume being larger in males than females (Mizukami, Nishizuka, & Arai, 1983). In P17
26 male rats, BLA is physically and functionally connected with the infralimbic PFC (Ganella,
27 Nguyen, Lee-Kardashyan, Kim, Paolini, & Kim, 2018; Santarelli, Khan, & Poulos, 2018) but
28 not the hippocampus (Ganella et al., 2018; Li, Gao, & Zhou, 2018). It would be critical to
29 determine BLA connectivity with PFC or hippocampus in P17 female rodents.

30 The present study reports a novel dissociation between context-dependent expression
31 of fear conditioning and extinction in developing female rats, with the emergence of context-
32 specific fear extinction preceding context conditioned fear. Notably, sex differences in the
33 underlying molecular and neural mechanisms of context learning in juvenile rodents still

1 remain largely unknown, because previous studies commonly pooled sexes (Travaglia et al.,
2 2016) or only used male rodents (e.g. Kim, Hamlin, & Richardson, 2009). With increasing
3 evidence for prepubertal sex differences in context-dependent memories (Cimadevilla et al.,
4 1999; Park et al., 2017b; Roof, 1993a; Short et al., 2016, 2017) future developmental studies
5 should examine sexes separately or together but with enough number of animals per sex to
6 ensure power to detect sex differences.

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Table 1. Mean $\pm$ SEM baseline freezing levels for all experiments

Experiment	Group	Conditioning	Extinction	Test	n
1	P18-Same	1.7 $\pm$ 0.9*	26.3 $\pm$ 9.2	4.1 $\pm$ 2.8	12
	P18-Different	2.8 $\pm$ 0.7*	29.4 $\pm$ 8.1	21.2 $\pm$ 4.5**	15
	P25-Same	0.7 $\pm$ 0.5	31.0 $\pm$ 6.5	16.4 $\pm$ 6.2	14
	P25-Different	1.1 $\pm$ 0.3	30.4 $\pm$ 4.6	27.0 $\pm$ 7.1**	14
2	P18 Not weaned-Immediate	1.4 $\pm$ 0.6		4.6 $\pm$ 3.2	15
	P18 Not weaned-24 hours	2.3 $\pm$ 0.7		5.3 $\pm$ 2.8	15
	P25 Not weaned-Immediate	2.9 $\pm$ 1.3		5.7 $\pm$ 3.0	14
	P25 Not weaned-24 hours	0.9 $\pm$ 0.3		4.3 $\pm$ 2.3	12
	P25 Weaned-Immediate	0.6 $\pm$ 0.4		0.8 $\pm$ 0.5	12
	P25 Weaned-24 hours	1.1 $\pm$ 0.4		1.0 $\pm$ 0.5	10

*Significant main effect of Age ($p < 0.05$)

**Significant main effect of Test Context ($p < 0.05$)

Table 2. Summary of context fear and renewal in developing male and female rats

	Male rats (Park, Ganella & Kim, 2017a)		Female rats (present study)	
	18 days of age	25 days of age	18 days of age	25 days of age
Renewal	Absent	Present	Present	Present
Context memory	Comparable		25 days of age > 18 days of age	

FIGURE LEGEND

Figure 1. Mean ($\pm$ SEM) CS-elicited % freezing. In experiment 1, rats showed comparable CS-elicited freezing during (A) conditioning and (B) extinction. (C) All rats displayed

1 renewal when tested in a different context to extinction (#main effect of Test Context, $p <$
2 0.05). P25 rats froze more than P18 rats (*main effect of Age, $p < 0.05$). In Experiment 2, (D)
3 rats showed comparable CS-elicited freezing during conditioning. (E) At immediate context
4 test, P25-weaned (W) and –not weaned (NW) groups showed significantly higher contextual
5 freezing compared to P18-not weaned (NW) group (post hoc tests * p s < 0.05). (F) While all
6 rats showed increased cue-elicited freezing at 24 hours compared to immediate test (#main
7 effect of Test Interval, $p < 0.05$), weaning had no effects.

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