

Post-retrieval extinction in adolescence prevents return of juvenile fear

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Traumatic experiences early in life can contribute to the development of mood and anxiety disorders that manifest during adolescence and young adulthood. In young rats exposed to acute fear or stress, alterations in neural development can lead to enduring behavioral abnormalities. Here, we used a modified extinction intervention (retrieval+extinction) during late adolescence (post-natal day 45 [p45]), in rats, to target auditory Pavlovian fear associations acquired as juveniles (p17 and p25). The effects of adolescent intervention were examined by assessing freezing as adults during both fear reacquisition and social transmission of fear from a cagemate. Rats underwent testing or training at three time points across development: juvenile (p17 or p25), adolescent (p45), and adult (p100). Retrieval+extinction during late adolescence prevented social reinstatement and recovery over time of fears initially acquired as juveniles (p17 and p25, respectively). Adolescence was the only time point tested here where retrieval+extinction prevented fear recall of associations acquired 20+ days earlier.

[Supplemental material is available for this article.]

Adolescence is a time of rapid developmental change that encompasses the period of puberty and the transition out of childhood toward sexual maturity. In humans, adolescence marks a sensitive time frame in which the risk of depression as well as other psychopathologies increases dramatically compared with childhood (Hankin et al. 1998; Kessler et al. 2001; Nemeroff et al. 2006). Although not always the case, the occurrence of childhood trauma in adolescent clinical cases of depression (Gunnar 2003; Nemeroff et al. 2006) and antisocial behavior (Jonson-Reid et al. 2010; Perepletchikova and Kaufman 2010; Haller et al. 2014) is high and treatment efficacy in depressed individuals with childhood trauma differs from those without early life trauma exposure (Nemeroff et al. 2003).

In the laboratory setting, cued Pavlovian fear conditioning is a frequently used animal model of fear learning. The neural circuitry necessary for associating a previously neutral conditioned stimulus (CS) with an aversive unconditioned stimulus (US) is well understood and these pathways overlap greatly with those involved in anxiety disorders, emotional learning, and the stress response in general. Once learned, the fear response is extremely persistent. Reducing fear with techniques thought to target the reconsolidation of a reactivated memory (Przybylski et al. 1999) shows promise in both the laboratory (Misanin et al. 1968; Land et al. 2000; Nader et al. 2000; Sara 2000; Rao-Ruiz et al. 2011) and clinical setting (Brunet et al. 2008). In addition, the repeated presentation of a feared cue (extinction) during the time that the memory is reactivated (retrieval+extinction) has been shown to be more effective than standard extinction in persistently reducing fear in rodents (Monfils et al. 2009; Clem and Haganir 2010; Rao-Ruiz et al. 2011; Xue et al. 2012; Gräff et al. 2014; Sartor and Aston-Jones 2014; but see also Chan et al. 2010) and humans (Schiller et al. 2010; Agren et al. 2012; Xue et al. 2012; Steinfurth et al. 2014). Older or stronger memories, however, are more difficult to disrupt than recently formed mem-

ories (Milekic and Alberini 2002; Suzuki et al. 2004; Wang et al. 2009; Robinson and Franklin 2010) and the retrieval+extinction protocol alone does not seem to target more remote (30-d-old) fear memories acquired during adulthood (Costanzi et al. 2011; Gräff et al. 2014).

The extended developmental trajectory of the pathways that underlie fear learning (e.g., prefrontal-amygdala connectivity) (Spear 2000; Gogtay et al. 2004; Suzuki et al. 2005) makes them especially sensitive to environmental disruption across development (Lupien et al. 2009; Tottenham 2014). Fear memories acquired very early in life are typically subject to infantile amnesia (Wetzler and Sweeney 1986) and forgotten within a matter of days (Campbell and Campbell 1962; Callaghan and Richardson 2012). Rats fear conditioned at p17 show expected fear responses to the conditioned stimulus (e.g., freezing and vocalizing) but do not retain the information for longer than 10 d after fear conditioning (Callaghan and Richardson 2012). Despite the inability to explicitly recall the previously learned fear association, enduring changes in the fear circuitry are still observed as the animal ages (Li and Richardson 2013). As young rats are weaned from their mother, they begin to transition to a more adult-like pattern of learning. Rodents fear conditioned at p24, for example, form explicit fear associations that are remembered into adulthood (Akers et al. 2012).

Here, we attempted to target specific CS–US associations acquired early in life (p17 or p25) using either standard or post-retrieval extinction during late adolescence (p45) in rats. In four separate experiments, we examined the efficacy of these behavioral interventions (standard extinction and retrieval+extinction) in targeting remote fear memories acquired early in life by examining adult fear expression and reacquisition (see Table 1 for procedural timeline). Fear relearning in adults was measured with

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Table 1. Overview of experimental timeline

	Fear Experience #1					Fear Experience #2			
	Direct FC	Naïve	(Days elapsed)	Intervention	(Days elapsed)	Direct FC	Social FC (FCbP)	No FC (housing exposure only)	LTM test
Exp. 1	p17	p17	(28)	p45	(55)	Adult	Adult	Adult	Y
Exp. 2	p25	p25	(20)	p45	(55)	Adult	Adult	Adult	Y
Exp. 3	p25	p25	(60)	Adult	(55)	Adult	N/A	N/A	Y
Exp. 4	Adult	Adult	(20)	Adult	(55)	Adult	N/A	N/A	Y

In all experiments, rats were exposed to an initial fear experience followed, several days later, by a behavioral intervention, and then exposed to a second fear experience 55 d later. In Experiments 1 and 2, rats were either fear conditioned or left Naïve as juveniles (p17 or p25) and underwent behavioral interventions at p45 aimed at reducing the juvenile fear memory (retrieval+extinction or standard extinction without retrieval). As adults (~p100), each triad underwent the fear conditioning by proxy paradigm. Experiments 3 and 4 followed a similar timeline except all interventions were performed as adults and instead of fear conditioning by proxy all rats were reconditioned directly. Behavior was considered most relevant at the beginning and end of the extinction intervention, the first CS exposure after intervention, and long-term memory retention tests.

either direct Pavlovian fear conditioning or through a socially transmitted fear paradigm (fear conditioning by proxy; see Fig. 1 for design) previously shown to produce a wider range of behavioral responses, including freezing, and which may provide a more sensitive range of detection than direct fear conditioning (Bruchey et al. 2010; Jones et al. 2014).

Results

In Experiments 1 and 2, we investigated whether p45 standard extinction or retrieval+extinction of juvenile (p17 [Experiment 1] or p25 [Experiment 2]) fear memories could attenuate fear responding in adults and influence either direct or indirect (e.g., social) fear relearning. In Experiments 3 and 4, we investigated whether adult standard extinction and/or retrieval+extinction of either p25 (Experiment 3) or remote adult (Experiment 4) fear memories could attenuate fear responding at a later time and influence direct fear relearning.

For ease of understanding, we labeled the behavioral groups as “Fear Experience #1; extinction intervention; Fear Experience #2” with predetermined time points in development assigned for each experiment (see Table 1 for experimental outline).

Adolescent retrieval+extinction of remote (>20 d) explicit fear associations prevents adult fear recall

Fear recall was assessed by comparing freezing levels to the CS several days after intervention (standard extinction or retrieval+extinction). In all experiments, this was the first CS of direct adult fear conditioning (immediately prior to the delivery of the US).

Fear recall during CS 1 of Adult FC

Experiment 1: p17 FC–p45 intervention. Intervention at p45 (extinction vs. retrieval+extinction) in rats that were fear conditioned at p17 resulted in no significant differences in freezing between groups when tested for fear recall as adults (p90–p100) ($F_{(3,23)} = 1.03$, $P = 0.4$) (Fig. 2A). It is important to note that there was very little retention of the CS–US association when first tested as adults (~20%) and all groups froze at levels similar to that of naïve rats (consistent with the low levels of freezing seen during adolescence after p17 conditioning; Supplemental Fig. S1a).

Experiment 2: p25 FC–p45 intervention–adult FC. Intervention at p45 in rats that were fear conditioned at p25 differentially affected fear recall as adults (overall ANOVA: $F_{(3,34)} = 8.86$, $P < 0.001$). Effectively, those that received retrieval+extinction froze significantly less at recall than those in either the standard extinction (Tukey: $P = 0.006$) or no extinction groups ($P = 0.021$) and were not significantly different than naïve rats ($P = 0.945$) (Fig. 2B). Naïve–Naïve rats froze significantly less than both FC-Std Ext ($P = 0.001$) and FC-No Ext ($P = 0.005$). There were no differences in freezing to the first fear-conditioning cue in rats that were exposed to standard extinction during adolescence (FC-Std Ext) or nothing at all (FC-No Ext) ($P = 0.956$) (Fig. 2B).

Experiment 3: p25 FC–adult intervention–adult FC.

Adult intervention did not prevent fear recall of a p25 fear memory. Between

group analysis of freezing to the first cue of fear conditioning revealed a significant effect of previous fear experience ($F_{(3,40)} = 11.56$, $P < 0.001$) with naïve rats freezing significantly less than all other fear experienced rats, even after interventions (all P s < 0.01) (Fig. 2C).

Experiment 4: adult FC–adult intervention–adult FC. None of the adult behavioral interventions prevented fear recall of a remote fear memory acquired as an adult. Between group analysis of freezing

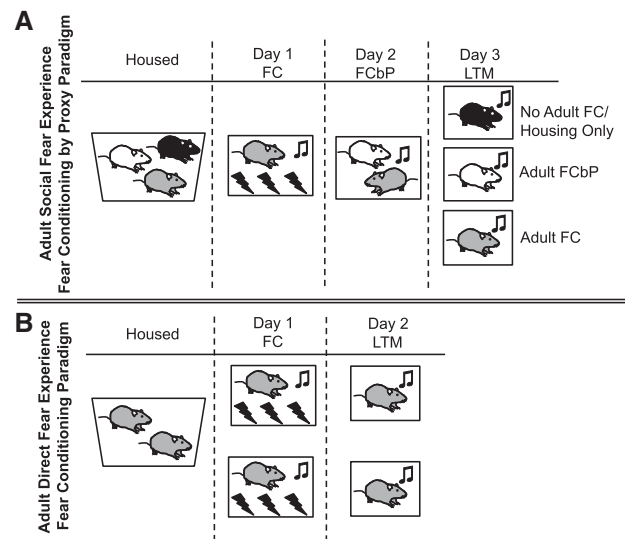


Figure 1. Fear Experience #2 experimental design (adult). (A) Social fear exposure—rats are housed in triads. On Day 1, one rat of the triad is fear conditioned. On Day 2, the fear-conditioned rat (Adult FC) and a cage mate (Adult FCbP) are returned to the chamber and the CS is played. This session is called fear conditioning by proxy (FCbP). On Day 3, long-term memory is tested by placing each rat in the chamber individually, including a No Adult FC rat that is socially exposed to fear in adulthood through housing, and presenting the CS. All the triads of rats in Experiments 1 and 2 underwent this fear conditioning by proxy paradigm as adults. (B) Direct fear exposure—rats are socially housed in dyads. On Day 1, both rats are directly fear conditioned. On Day 2, the CS is played in the absence of the US as a long-term memory test of the fear association.

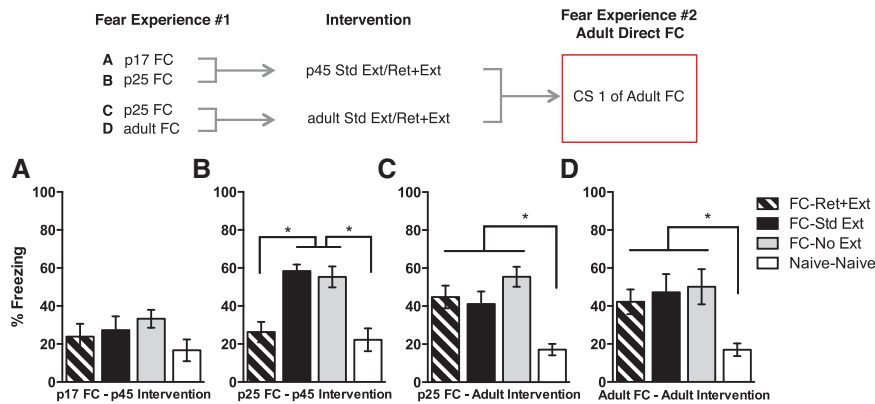


Figure 2. Fear recall in adults—freezing to the first CS of adult FC. (A) After p17 FC, adult rats froze at the same levels as rats that were left naïve at p17 ($n = 6$), regardless of extinction intervention at p45 (ret+ext: $n = 7$; std ext: $n = 8$; no ext: $n = 6$). (B) After p25 FC, adult rats did recall the fear association (no ext ($n = 10$) vs. naïve ($n = 9$) $P < 0.05$) and p45 ret+ext ($n = 9$) reduced freezing in adults compared with std ext ($n = 10$; $P < 0.05$) in line with naïve animals. (C, D) When the extinction intervention was applied as adults after either p25 FC (C) or adult FC (D) neither ret+ext (p25 FC: $n = 10$; adult FC: $n = 8$) nor std ext (p25 FC: $n = 10$; adult FC: $n = 6$) reduced fear recall compared with no ext (p25 FC: $n = 10$; adult FC: $n = 4$) (both P s > 0.05) and all previously fear-conditioned rats froze more than naïve rats (p25 FC: $n = 14$; adult FC: $n = 8$) (all P s > 0.05). Error bars \pm SEM. (*) $P < 0.05$.

to the first cue of fear conditioning revealed a significant effect of previous fear experience ($F_{(3,22)} = 5.27$, $P = 0.007$) with naïve rats freezing significantly less than all other fear-experienced rats, even after interventions (all P s < 0.05) (Fig. 2D).

Neither early life fear experience nor adolescent intervention interferes with retention after direct fear reconditioning as adults

Long-term fear retention of a direct fear association in adults was determined by measuring freezing to three CS presentations 24–48 h after direct adult fear reconditioning.

Long-term memory after adult fear reconditioning

Experiment 1: p17 FC–p45 intervention–FC. Despite some differences in freezing during acquisition (see Supplemental results; Fig. S1b), when adult fear-conditioned rats (Adult FC Rat) were tested for LTM (48 h after adult conditioning), there was no significant effect of adolescent intervention group on LTM freezing ($F_{(3,23)} = 0.422$, $P = 0.739$) in rats previously fear conditioned at p17 (Fig. 3A).

Experiment 2: p25 FC–p45 intervention–FC. P25 fear experience did not influence fear expression after direct reconditioning regardless of p45 intervention. There were no significant differences in LTM freezing between groups ($F_{(3,33)} = 0.481$, $P = 0.697$) in rats that were fear conditioned directly as adults (Fig. 3B).

Adult retrieval+extinction may expedite fear reduction after direct fear reconditioning

Long-term memory after adult fear reconditioning

Experiment 3: p25 FC–adult intervention–adult FC. When p25 fear conditioning was followed by adult intervention, rats retained the fear memory after direct reconditioning; however, adult retrieval+extinction rats showed a rapid decrease of freezing after repeated CS presentation during the LTM test the next day. After fear reconditioning, the effect of prior experience on freezing to the three CS presentations of the long-term

memory test approached significance (repeated measures ANOVA $F_{(3,40)} = 4.204$, $P = 0.054$). Follow-up planned comparisons revealed that the rats conditioned at p25, which received retrieval+extinction in adolescence, and were reconditioned in adulthood (FC-Ret+Ext-FC rats) froze significantly less than the p25 conditioned rats that did not receive extinction in adolescence (FC-No Ext-FC) (post hoc Tukey $P = 0.041$) (Fig. 3C). This effect was driven by freezing to the last CS of the LTM test (Cue 3 $F_{(3,40)} = 5.42$, $P = 0.003$), in which FC-Ret+Ext-FC rats froze significantly less than both FC-No Ext-FC rats ($P = 0.01$) and Naïve–Naïve-FC rats ($P = 0.006$) (Supplemental Fig. S3c).

Experiment 4: adult FC–adult intervention–adult FC.

When fear conditioning and intervention (extinction vs. retrieval+extinction) took place as adults, all rats retained the fear memory after direct reconditioning;

however, the retrieval+extinction group showed a rapid decrease of freezing after repeated CS presentation (Supplemental Fig. S4c). When tested for LTM the next day, there was a significant effect of prior experience on freezing to the three CS presentations (repeated measures ANOVA $F_{(3,22)} = 4.204$, $P = 0.017$) with FC-Ret+Ext rats freezing significantly less than FC-No Ext rats ($P = 0.03$). Over the entire LTM session, the Ret+Ext rats approached a significant decrease in freezing compared with the Std Ext ($P = 0.087$) and the previously naïve rats (directly fear conditioned as adults) ($P = 0.058$) (Fig. 3D).

Adolescent retrieval+extinction prevents potentiated adult social fear learning caused by juvenile fear conditioning

Adult social fear learning was measured in Experiments 1 and 2 with the fear conditioning by proxy paradigm (described in Fig. 1A). Freezing to the CS was analyzed in rats 24 h after exposure to a conspecific displaying fear to the CS (FCbP) or in rats that lived in the same cage but had no adult experience with the CS or US (No Adult FC/housing control). All rats, except those indicated as Naïve–Naïve, were fear conditioned to the same CS as juveniles (p17 or p25) and underwent an extinction intervention (retrieval+extinction, standard extinction, or no extinction) at p45.

Long-term memory after adult fear social exposure

Experiment 1: p17 FC–p45 intervention–adult social exposure. Adult FCbP rats (Day 3): Social exposure to a fearful rat was sufficient to increase freezing in rats that were fear conditioned at p17 followed by p45 standard extinction or no extinction. There was a significant effect of adolescent treatment group on LTM freezing in the FCbP rats ($F_{(3,23)} = 7.787$, $P = 0.001$). Follow-up Tukey HSD mean comparisons showed that retrieval+extinction at p45 significantly reduced the amount of freezing acquired through the fear conditioning by proxy paradigm compared with standard extinction ($P = 0.032$) and no extinction ($P = 0.002$) and was not significantly different than animals that had never been fear conditioned as juveniles ($P = 0.963$). FCbP rats that were fear conditioned at p17 and had no intervention at

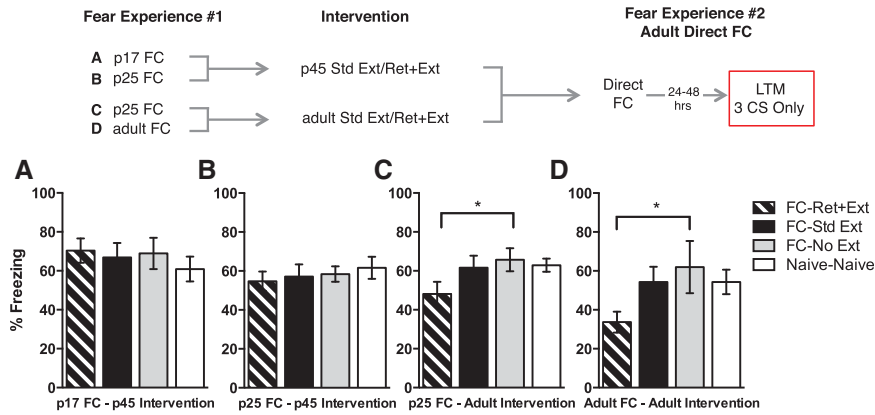


Figure 3. Freezing at LTM after adult direct FC. Neither p17 (A) nor p25 (B) juvenile fear conditioning influenced fear retention after direct adult relearning (all P s $>$ 0.05). Adult retrieval+extinction of p25 fear memories (C) and adult fear memories (D) moderately, but significantly reduced fear retention after direct relearning compared with no extinction (both P s $<$ 0.05), driven by a rapid decrease in freezing across the three LTM cues (see Supplemental Figs. S3C, S4C). Freezing across the three LTM cues were averaged each rat above. Group ns are the same as Figure 2. Error bars \pm SEM. (*) P $<$ 0.05.

p45 (FC-No Ext-FCbP) froze significantly more than naïve rats that underwent fear conditioning by proxy (Naïve-Naïve-FCbP) (P = 0.008) (Fig. 4A). A priori planned comparisons revealed that FCbP rats fear conditioned at p17 with no p45 extinction (FC-No Ext-FCbP) froze more than their home cage control counterparts (FC-No Ext-No FC) ($t_{(10)} = 2.4$, P = 0.04).

No adult FC rats (Day 3): There were no significant effects of treatment group on rats that were not fear conditioned again as adults and served as home cage controls for social fear exposure ($F_{(3,23)} = 1.1$, P = 0.37) (Fig. 4A).

Experiment 2: p25 FC-p45 intervention-adult social exposure. Adult FCbP rats (Day 3): Adolescent retrieval+extinction prevented reinstatement of freezing after exposure to a fearful conspecific in rats fear conditioned at p25. There was a significant effect of treatment group on freezing displayed by the FCbP rat during LTM tests ($F_{(3,32)} = 5.713$, P = 0.003). Post hoc Tukey mean comparisons revealed FC-Ret+Ext-FCbP rats froze less after FCbP than FC-Std Ext-FCbP rats (P = 0.041) and FC-No Ext rats (P = 0.003). Naïve rats that underwent FCbP as adults were not statistically different than either the FC-Ret+Ext-FCbP rats (P = 0.715) or the FC-Std Ext-FCbP rats (P = 0.383) and only approached significance compared with the FC-No Ext rats (P = 0.059) (Fig. 4B).

No adult FC rats (Day 3): p45 retrieval+extinction prevented fear recall in adults that were not fear conditioned a second time. There was a significant effect of treatment group on freezing displayed by the No Adult FC rat during LTM tests ($F_{(3,28)} = 8.447$, P $<$ 0.001). Follow-up Tukey mean comparisons revealed that both the FC-Ret+Ext-No FC and Naïve-Naïve-No FC rats who were not fear conditioned as adults froze significantly less than both FC-No Ext-No FC and FC-Std Ext-No FC rats (all P s $<$ 0.03) (Fig. 4B).

Discussion

Our results show that late adolescence (p45) represents a time in development where intervention with retrieval+extinction can target remote fear associations acquired as juveniles and prevent fear recall as adults without interfering with later direct learning in the rat. Additionally, we show that juvenile fear conditioning potentiates social fear learning. Fear conditioning by proxy was sufficient to reinstate fear to juvenile (p17) fear memories in rats that did not receive retrieval+extinction during adolescence.

The differences in freezing observed during adult fear conditioning after adolescent intervention, in both Experiments 1 and 2, disappeared when the rats were tested for freezing to the cue on subsequent days suggesting that either differences in fear acquisition in the presence of a threat (US) not in fear expression to the CS itself or the final US administration was sufficient to abolish any group differences in freezing during acquisition. Adult retrieval+extinction resulted in no differences in freezing during reacquisition (in the presence of the US), but did show a rapid decrease in freezing during long-term memory tests following reacquisition. This may suggest that adult retrieval+extinction of remote fear memories do not entirely target the original fear memory but rather results in a more readily retrieved extinction memory during later CS presentation.

In order to begin to disentangle the role of *developmental* age of the memory or intervention from *overall* age of the memory (time between interventions and tests) on adult reconditioning, two experiments were performed in adult animals (Experiments 3 and 4). The timing between interventions remained similar to the p25 developmental experiment (Experiment 2); however,

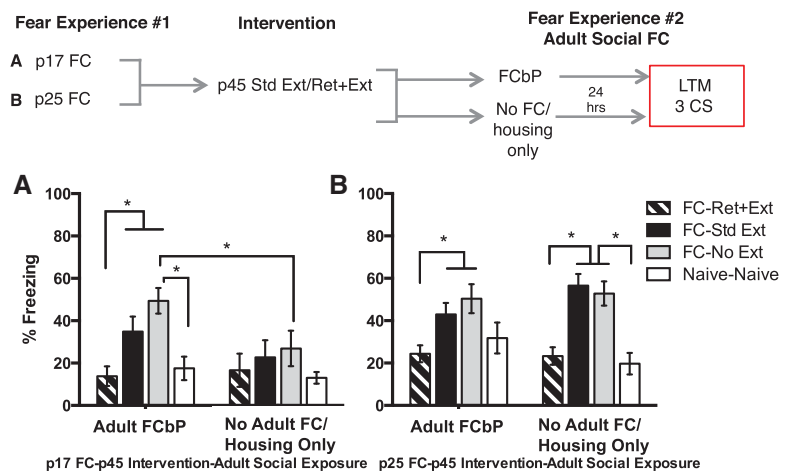


Figure 4. LTM after adult social fear exposure in rats with juvenile fear experience. (A) p17 fear conditioning potentiated social fear learning after fear conditioning by proxy (FC-No Ext-FCbP vs. FC-No Ext-No FC; P $<$ 0.05). p45 Ret+Ext prevented social fear reinstatement compared with Std Ext and No Ext (both P s $<$ 0.05). (B) p45 Ret+Ext after p25 FC prevented social fear reinstatement through FCbP (FC-Ret+Ext-FCbP vs. FC-Std Ext-FCbP and No Ext, P s $<$ 0.05) and reduced fear recall in rats not fear conditioned as adults and only housed with fearful conspecifics. Group ns for panels A and B are the same as in Figure 2A,B, respectively. Error bars \pm SEM. (*) P $<$ 0.05.

the interventions occurred in adult animals on CS–US associations acquired as either juveniles (Experiment 3) or adults (Experiment 4). In Experiment 4, we found that neither adult retrieval+extinction nor adult standard extinction of 20+d-old fear memories prevented recall of fear when tested for freezing 55 d later. These results are in line with other research on the efficacy of retrieval+extinction on remotely acquired fear memories (Gräff et al. 2014; Clem and Huganir 2010). Interestingly, when tested for 24 h after fear reacquisition, retrieval+extinction provided some resistance against the retention of this required fear memory. However, this effect size was moderate ($f = 0.613$) and seems to be driven by more rapid extinction over the three cues in retrieval+extinction rats. The rapid extinction during LTM can be interpreted in two ways: (1) retrieval+extinction destabilized the memory trace enough to prevent full retention of the reacquisition but not enough to update the memory as entirely safe and (2) retrieval+extinction did not target the original memory trace but instead allowed the animal to better discriminate between fear conditioning and extinction scenarios (Millan et al. 2013).

Adolescent retrieval+extinction did not influence retention of direct adult fear reacquisition, but did prevent the more indirect acquisition of socially transmitted fear. In rats that underwent retrieval+extinction during adolescence, fear conditioning by proxy did not reinstate fear to a CS fear conditioned at either p17 or p25. Therefore, retrieval+extinction during adolescence prevented both the recovery of fear during adulthood and reinstatement of that fear after exposure to a fear-conditioned cagemate but did not attenuate relearning the CS–US association through direct experience.

General discussion

The functional development of the amygdala is delayed until animals begin to become independent of their mother (Moriceau and Sullivan 2006; Thompson et al. 2008) and stressors early in life result in later life dysfunction (Huang and Lin 2006; Sevelinges et al. 2007; Kuramochi and Nakamura 2009; Moriceau et al. 2009; Raineki et al. 2012; Tzanoulinou et al. 2014). Connectivity between the prefrontal cortex and the amygdala is not refined until late in adolescence (approximately p30–p60) (Cunningham et al. 2002; Cressman et al. 2010; Koss et al. 2014). Prefrontal cortex–amygdala connections are essential for emotion regulation and it is believed that synaptic pruning in the prefrontal cortex, which is neither functionally nor morphologically mature until late adolescence (Van Eden and Uylings 1985), may be responsible for unmasking psychopathologies during adolescence (Feinberg 1982; Andersen and Teicher 2008; Cressman et al. 2010).

The current experiments provide unique insight into the boundary conditions of the retrieval+extinction paradigm and are interpretable in the context of direct or indirect reexposure to fear. Twenty-day-old explicit fear-conditioning memories can be targeted for recall by retrieval+extinction only during adolescence and not during adulthood but adult retrieval+extinction can impact relearning. Additional research is required to determine if p45 ret+ext is altering the developmental trajectory of the fear circuitry in rats fear conditioned as juveniles or if it is simply persistently targeting and reducing fear to the previously conditioned stimulus.

The neural mechanisms involved in extinction, and other fear reduction techniques, depend on the stage of development. Successful extinction of conditioned fear responses in adults is dependent on inhibition of the amygdala by the infralimbic cortex (Phelps et al. 2004; Quirk et al. 2006). During repeated CS exposure, animals form a new memory of the CS for extinction that is context dependent and expression is modulated by the hippo-

campus. During reconsolidation, it is presumed that the retrieval of a memory initiates a period of lability during which time the memory trace can be updated, strengthened, or blocked. Auditory fear memories undergo reconsolidation in the amygdala (Nader et al. 2000; Ben Mamou et al. 2006; Wang et al. 2009; Maddox and Schafe 2011). This is relevant from a developmental viewpoint because this circuitry in particular has an especially protracted developmental timeline. Unfortunately, at this time, very little work has been done on reconsolidation across development.

In a healthy, nonstressed environment, medial prefrontal cortical projections to the basal amygdala are pruned by ~50% in late adolescence (after p45) (Cressman et al. 2010). Inverse connectivity within the mPFC-amygdalar circuitry is essential for regulation of the extinction of conditioned fear and consolidation of extinction memories (Phelps et al. 2004; Quirk et al. 2006). Adolescent animals of multiple species, including humans, display extinction deficits as evidenced by both poor within-session extinction (Hefner and Holmes 2007; Johnson and Casey 2015) and later retention (McCallum et al. 2010; Kim et al. 2011; Pattwell et al. 2012; Baker et al. 2013). It has been postulated that the adolescent pruning of prefrontal synapses combined with the temporary increase in amygdala activity results in an inability of the prefrontal cortex, specifically the infralimbic cortex, to exert the necessary inhibitory control over the amygdala that leads to successful extinction (for review, see Baker et al. 2014).

When extinction procedures are manipulated, this impairment can be overcome. Kim et al. (2011) found that doubling the extinction training for adolescent rats resulted in adult-like retention of extinction the following day. Baker et al. (2013) found that targeting a recently acquired fear memory with variations of this retrieval+extinction paradigm resulted in a more effective reduction of fear in adolescent rats (p34–p37) compared with extinction paradigms that have no retrieval session. Johnson and Casey (2015) also found that extinction presented after retrieval in adolescent humans helped to overcome the extinction deficit typically present during adolescence. The results of the current experiment are in line with this research on adolescent extinction; however, the results presented here are unique in that they target fear memories acquired earlier in development and go on to examine the long-term consequence of extinction and fear reduction during adolescence. One major constraint of the extinction or retrieval+extinction sessions performed here is the lack of a test for extinction retention shortly after extinction (on p46). Although this leaves us in the dark about the immediate effectiveness of the fear-reducing procedures, long-term effects in adulthood were investigated providing perhaps a better measure of fear reduction and allowed us to avoid excessive repeated testing on the animals. Additionally, it is important to note that the housing conditions varied between Experiments 1–2 (rats housed in triads) and Experiments 3–4 (rats housed in dyads) given the nature of the fear conditioning by proxy design. While all animals were socially housed, the cages were identical in size between experiments and available living space/density within the cage, social hierarchy, and even home cage behavior after reconditioning (note that both rats in the cage were directly reconditioned in Experiments 3 and 4 compared with only one rat per triad in Experiments 1 and 2) could influence the stress response and the freezing measured here. Additionally, the efficacy of the retrieval+extinction manipulation decreases when higher numbers of rats are housed together (Kredlow et al. 2016) and should be taken into account when comparing freezing data from Experiments 1 and 2 to Experiments 3 and 4.

It is especially interesting that adolescent retrieval+extinction would prevent the recovery of juvenile fears when adult retrieval+extinction on fear memories of similar temporal

age fails to attenuate fear (Clem and Huganir 2010; Gräff et al. 2014). At the molecular level, retrieval+extinction of a fear memory targets synaptic plasticity within the lateral amygdala where it is hypothesized to destabilize the original memory trace (Monfils et al. 2009; Clem and Huganir 2010) by inducing a reconsolidation-like process in the LA, IL, and PRL (Tedesco et al. 2014), and engages a mechanism distinct from that involved in standard extinction (Lee et al. 2015). This suggests that successful fear reduction with the retrieval+extinction procedure requires destabilization of the memory trace in the LA paired with the incorporation of new information from the PFC. It stands to reason that if retrieval+extinction of an auditory CS relies mostly on the functioning of the amygdala, the transient increase in volume/activity that is specific to adolescence may allow for enhanced targeting of the fear memory unique to adolescence. Complimenting this idea, increases in the coupling of PFC-BLA activity specific to retrieval+extinction (Xue et al. 2012; Tedesco et al. 2014) may serve to further potentiate extinction-like fear reduction, including retrieval+extinction, during adolescence, when the circuitry is undergoing extensive synaptic remodeling, that update the previous fear memory as safe.

Boundary conditions of the retrieval+extinction paradigm have yet to be fully determined. These questions are mainly driven by seemingly minor procedural alterations that result in drastic changes in behavioral outcome (Chan et al. 2010; Flavell et al. 2011; Ma et al. 2012; Piñeyro et al. 2014). One possible explanation of these differences is to reason that the retrieval session must be salient enough to fully retrieve the memory, thus allowing destabilization of the entire memory trace (for review, see Auber et al. 2013; Piñeyro et al. 2014). Under this assumption, a single CS presentation of an older or stronger memory (Clem and Huganir 2010; Gräff et al. 2014); a partial presentation of compound cue (Jones et al. 2013); duration of retrieval: either too short of a retrieval session (Piñeyro et al. 2014) or too long of a retrieval session (Ishii et al. 2015); or even the context of the retrieval session or method of animal housing (Chan et al. 2010) may not allow for a complete retrieval of the memory trace, thus undermining the efficacy of behavioral update during reconsolidation. During periods of increased amygdala activity, such as during adolescence, CS presentation of a remote memory that may not typically induce reconsolidation might show enhanced ability to retrieve and target an otherwise impenetrable memory trace.

Further research is needed to determine the boundaries of the developmental timeline that are applicable to this method of fear reduction. The current set of experiments only looked at targeting fear memories acquired at two specific time points in development (p17, 4 d before pups are weaned from their mother and p25, 4 d after weaning). Memories formed either earlier or later in life may be subject to different constraints. Additionally, the definition of adolescence is a broad one and is subject to a number of confounding factors. Individual differences in hormonal and neural development are not taken into account here and despite attempts to perform all intervention at the same post-natal time point, there is no guarantee that all rats were at the same point in their development.

One possible interpretation of the current set of experiments is that heightened amygdala activity during adolescence results in more effective reconsolidation update mechanisms that rely on neuronal activity in the amygdala. Targeting early life experience

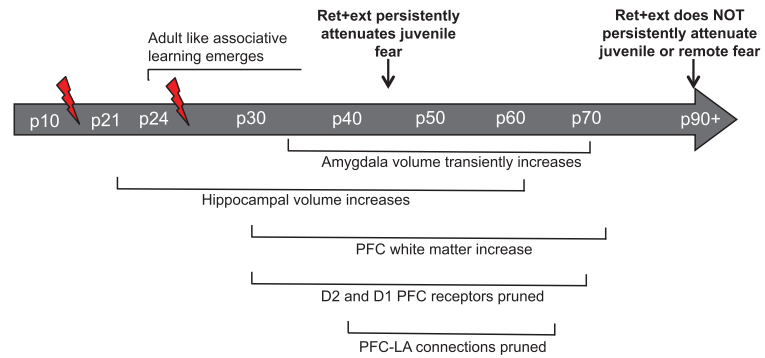


Figure 5. Summary of adolescent neural circuitry development and intervention results. Retrieval+extinction amidst the neuronal remodeling that occurs during adolescence persistently attenuates remote fear acquired as juveniles. Retrieval+extinction of remote fear memories in adult rats results in fear returning over time. (PFC) prefrontal cortex, (LA) lateral amygdala, (D) dopamine.

during adolescence, amidst dramatic neuronal remodeling (see Fig. 5 for timeline of neural development relevant to fear learning), may allow fear reduction techniques to more effectively target labile amygdala–prefrontal connections resulting in persistent modifications to the developing fear circuitry and possibly preventing some of the later emerging problems associated with early life fear experience.

Materials and Methods

General overview of design

Four experiments were conducted in order to examine how behavioral interventions on remotely acquired fear memories influenced freezing during a second fear experience several days after the intervention. The main question of our research was whether adolescent intervention could target juvenile fear memories and how this influenced either direct or indirect adult fear learning. Interventions performed in adult animals (3 and 4) were conducted following the same timeline to compare if observed effects on direct fear conditioning were due to passage of time or developmental age. Each experiment consisted of four main behavioral manipulations: Fear Experience #1 (either direct fear conditioning or no fear conditioning), an intervention to reduce fear (retrieval+extinction, standard extinction, no extinction), Fear Experience #2 (either direct or social fear exposure), and long-term memory tests for retention. The key difference between each experiment was the developmental age of the subject (see Table 1 for experimental timelines).

Subjects

In Experiments 1–3, subjects were male Sprague Dawley rats bred at The University of Texas at Austin. Male Sprague Dawley breeder rats (275–300g; Harlan) were paired with female Sprague Dawley rats (215–275g; from either Harlan or retained from previous breeding). Approximately 2 wk after pairing, the males were removed from the cage and females were checked daily for the presence of newborns. Rats were housed in clear plastic cages and maintained on a 12-h light–dark cycle (lights on at 0700 hours) with food (standard rat chow) and water provided ad libitum. On post-natal day 21 (p21) rats were weaned into either same sex triads (Experiments 1 and 2) or dyads (Experiment 3) with littermates assigned to the same behavioral treatment group.

In Experiment 4, subjects were male Sprague Dawley rats ordered directly from the vendor (275–300 g, Harlan) and pair housed for 1 wk prior to the start of experimental testing.

Procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

Eighty-two rats were used in Experiment 1, 107 rats were used in Experiment 2, 44 rats were used in Experiment 3, and 26 rats were used in Experiment 4.

Apparatus and stimuli

Fear Experience #1 took place in standard-conditioning chambers equipped with two metal walls, two plexiglass walls, and stainless-steel rod floors designed for mice connected to a shock generator (Coulbourn Instruments). Chambers were enclosed in acoustic isolation boxes (Coulbourn Instruments) and illuminated with a red light. Behavior was recorded with digital cameras mounted on the top of each unit. The chambers were wiped with water between each session. Stimulus delivery was controlled using Freeze Frame software (Coulbourn Instruments). The conditioned stimulus (CS) was a white noise (80 dB) 20 sec in duration and the unconditioned stimulus (US) was a 0.6 mA footshock, 500 msec in duration.

Intervention and fear Experience #2 took place in the left portion of a closed shuttle box chamber equipped with two metal walls, two black and white striped walls, stainless-steel rod floors (designed for rats) connected to a shock generator and enclosed in acoustic isolation boxes (Coulbourn Instruments). Light peppermint scent was added to the pans below the floors to further change the context compared with the fear Experience #1 context (ABB design). Behavior was recorded using digital cameras mounted on the top of each unit. Stimulus delivery was controlled using Graphic State 2 software (Coulbourn Instruments). The CS and US were identical to those used during the previous fear experience.

Behavioral methods

Fear Experience #1

On p17 (Experiment 1), p25 (Experiments 2 and 3), or 1 wk after arrival as adults (Experiment 4) rats were either directly fear conditioned or remained naïve, as described below.

Direct fear conditioning: Juvenile rats were removed from their mother, placed in an identical, but clean, cage lined with cedar chips, and transported to the fear-conditioning room. Already weaned or adult rats were transported in their home cage to the fear-conditioning room. After a 10-min habituation period, each rat received three 20-sec presentations of an 80 dB white noise CS each coterminating with a 500 msec 0.6 mA footshock.

Naïve: p17 rats in the Naïve group were removed from their mother, placed in a clean cage, and transported to the behavioral rooms where they remained in the transportation cage for the same amount of time as a fear-conditioning session before being returned to their mothers. p25 and adult Naïve rats were transported in their home cage to the fear-conditioning room and left undisturbed for the same amount of time. The naïve rats were exposed to neither the CS nor the US.

Intervention

Behavioral intervention was performed in a different context (Context B) than the original fear-conditioning context (Context A) between 20 and 60 d after fear Experience #1, as described in Table 1. Entire cages were assigned to one of the three intervention groups below. Naïve rats that did not receive any fear conditioning for fear Experience #1 remained naïve during the intervention stage as well (Naïve–Naïve) and were not exposed to conditioned stimuli.

Retrieval+extinction (FC-Ret+Ext): On p45 (Experiments 1 and 2) or as adults (Experiments 3 and 4), rats in the FC-Ret+Ext group received a single retrieval of the CS (20 sec, 80 dB WN) after a 2-min habituation period and were promptly removed from the chambers and returned to the home colony for 1

h. Care was taken to avoid disruption during this 1-h period (cage changes, room entries, etc.). The rats were then returned to the chambers where they received 18 CS presentations (variable ITI, mean = 180 sec).

Standard extinction (FC-Std Ext): Rats in the FC-Std Ext group were exposed to 19 nonreinforced CS presentations (to equate CS exposure) (variable ITI, mean = 180 sec).

No Extinction (FC-No Ext): Rats in the FC-No Ext group were not disturbed and remained in their home cage on p45.

Fear Experience #2

Indirect fear experience: fear conditioning by proxy (Experiments 1 and 2). In Experiments 1 and 2, each triad of adult rats underwent a social fear learning paradigm described in detail in Bruchey et al. (2010) and Jones et al. (2014). On Day 1, one rat of each triad was fear conditioned to a noise paired with a footshock. On Day 2, the fear-conditioned rat (Adult FC rat) was returned to the fear-conditioning chamber accompanied by a cagemate (Adult FCbP rat) and the noise was played in the absence of the footshock. The third rat (No Adult FC rat) remained in the home cage and on Day 2 was allowed to freely interact with the fear conditioned (Adult FC) and fear conditioned by-proxy (Adult FCbP) rat when they were returned after the fear conditioning by-proxy session on Day 2. The following day (Day 3), all rats (Adult FC, Adult FCbP, and No Adult FC) were placed in the chambers alone and tested for fear expression (freezing) to the noise (see Fig. 1 for fear conditioning by proxy design).

Fear conditioning (FC; Adult Day 1): On the fear-conditioning day, after a 7-min habituation period, one rat per triad received three presentations of the CS (duration = 20 sec, ITI = 180 sec on average, variable), each coterminating with the US (intensity = 0.6 mA; duration = 500 msec).

Fear conditioning by proxy (FCbP; Adult Day 2): One day after conditioning, the fear-conditioned rat was returned to the chamber accompanied by a previously naïve cagemate (now termed the Adult FCbP rat). The rats were allowed to freely interact while the CS was presented three times (ITI = 180 sec on average, variable). The third rat of the triad (No Adult FC rat) remained in the home cage.

Long-term memory test (LTM; Adult Day 3): Twenty-four hours after fear conditioning by proxy, each rat (Adult FC, Adult FCbP, and No Adult FC) was placed in the chamber alone and received three presentations of the CS to determine fear to the noise (ITI = 180 sec on average, variable). Unless otherwise noted, fear retention at LTM was analyzed as an average of the three CS presentations for each rat.

Direct fear conditioning (Experiments 3 and 4). In Experiments 3 and 4, all rats in a cage underwent direct fear conditioning as described in Adult Day 1, above. Twenty-four hours later long-term memory was tested as described in Adult Day 3, above.

Data scoring and analysis

Freezing was defined as the absence of any movement, excluding breathing and whisker twitching. The total number of seconds spent freezing throughout the CS presentation is expressed as a percentage of CS duration (20 sec) for analysis. ANOVAs were performed on each adult fear-conditioning group (Adult FC, Adult FCbP, or No Adult FC) using juvenile-adolescent treatment (FC-Ret+Ext, FC-Ext, FC-No Ext, and Naïve–Naïve) as the between-subjects factor. Significant main effects were followed up with Tukey post hoc mean comparisons where appropriate.

Freezing during adult fear conditioning was analyzed with a repeated measures ANOVA (since the occurrence of the footshock after each cue makes averaging values inappropriate) with fear-conditioning cue as the within-subjects factor and juvenile-adolescent treatment as the between-subjects factor. In this

paradigm, freezing to each cue of fear conditioning, although influenced by the immediate threat of the US, provides important information. Freezing during the very first cue of the adult fear-conditioning session provides a measure of what information the animal has retained from the first fear-conditioning experience (because this is the last time the rat was conditioned) as well as provides an indicator of long-term (~55 d) efficacy of the adolescent intervention.

The endpoints of interest were recall of fear when the CS was presented for the first time after interventions (first cue of FC in fear Experience #2) and group differences in freezing during long-term memory tests after a second fear experience. Spontaneous recovery of freezing was determined with a paired samples *t*-test comparing freezing during the last three cues of standard extinction or retrieval+extinction with freezing during the first cue of adult fear conditioning.

Competing interest statement

The authors disclose no competing interests.

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