Research

Behavioral and immunohistochemical characterization of rapid reconditioning following extinction of contextual fear

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A fundamental property of extinction is that the behavior that is suppressed during extinction can be unmasked through a number of postextinction procedures. Of the commonly studied unmasking procedures (spontaneous recovery, reinstatement, contextual renewal, and rapid reacquisition), rapid reacquisition is the only approach that allows a direct comparison between the impact of a conditioning trial before or after extinction. Thus, it provides an opportunity to evaluate the ways in which extinction changes a subsequent learning experience. In five experiments, we investigate the behavioral and neurobiological mechanisms of postextinction reconditioning. We show that rapid reconditioning of unsignaled contextual fear after extinction in male Long–Evans rats is associative and not affected by the number or duration of extinction sessions that we examined. We then evaluate c-Fos expression and histone acetylation (H4K8) in the hippocampus, amygdala, prefrontal cortex, and bed nucleus of the stria terminalis. We find that in general, initial conditioning has a stronger impact on c-Fos expression and acetylation than does reconditioning after extinction. We discuss implications of these results for theories of extinction and the neurobiology of conditioning and extinction.

One of the most theoretically important findings from research on Pavlovian conditioning is that extinction suppresses behavior without necessarily altering the original association. Several pieces of behavioral evidence support this, including findings that after extinction, the behavior will return with time (spontaneous recovery), changes in context (contextual renewal), or reexposure to the unconditioned stimulus (reinstatement). The fourth piece of evidence for extinction as a suppressive process is that behavior is rapidly reestablished after fewer conditioning trials than are needed to establish the initial behavior (Bouton et al. 2012; Goode and Maren 2014). This rapid reconditioning effect is robust but is generally less studied relative to the other unmasking procedures and little is known about the neurobiology of postextinction reconditioning.

Studies of reconditioning after extinction have revealed that the amount of reconditioning can be influenced by the amount of extinction. For example, following relatively brief extinction, reconditioning occurs more quickly compared to initial conditioning, but following extensive extinction, this effect may be eliminated or reconditioning may be even slower compared to initial conditioning (e.g., Pavlov 1927; Bouton 1986; Bouton and Swartzentruber 1989; Hart et al. 1995; Rescorla 2001; Leung et al. 2007; Williams and Lattal 2019). Although reconditioning has been used as a tool to assess the strength of extinction learning in the face of neurobiological manipulations (Raybuck et al. 2013; Bolkan and Lattal 2014; Hart et al. 2014), the neurobiology of reconditioning itself is understudied compared to other postextinction unmasking phenomena. Reconditioning is a particularly nice experimental approach for comparing initial conditioning with the postextinction expression of behavior because the conditions during initial conditioning and postextinction reconditioning can be closely matched. Thus, the impact of the same

number of conditioning trials can be assessed in groups that are receiving those trials for the first time or for the second time (after extinction).

There is some reason to think that the circuits and molecular mechanisms that underlie initial conditioning and postextinction reconditioning, in general, may differ (e.g., Motanis and Maroun 2012; Perry and McNally 2012). Recent work on fear conditioning and extinction has focused on the extended amygdala, hippocampus, and prefrontal cortex. There are many demonstrations that mechanisms of learning in these regions may be different as a function of behavioral history prior to an associative learning experience. For example, preexposure to a context can change the hippocampal and amygdala requirements of contextual fear conditioning (e.g., Huff et al. 2005). A second fear conditioning session recruits different mechanisms from a first conditioning trial (e.g., Kim and Davis 1993; Maren et al. 1996; Anglada-Figueroa and Quirk 2005) and postextinction reconditioning involves overlapping and distinct molecular mechanisms in the amygdala compared to initial acquisition (Laurent and Westbrook 2009; Motanis and Maroun 2012). The role of the subregions of the medial prefrontal cortex (mPFC) has been mixed, with demonstrations that tetanic stimulation of the mPFC (Deschaux et al. 2011; Zheng et al. 2013) or inactivation of the prelimbic cortex (PL) impairs or enhances postextinction reconditioning in different tasks (Willcocks and McNally 2013; Fu et al. 2016) and other preparations similarly showed mixed effects of PL and infralimbic cortex (IL) inactivation on reacquisition. Finally, many studies have found that the anterior BNST (Alheid and Heimer 1988), a member of the extended amygdala that shares similar connections and

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neuroanatomical makeup with the CeA (Alheid et al. 1995) plays a selective role in aspects of fear conditioning (Lee and Davis 1997; Walker and Davis 1997; Schulz and Canbeyli 1999; Sullivan et al. 2004; Waddell et al. 2006; Davis et al. 2010; Zimmerman and Maren 2011) and reinstatement (Erb and Stewart 1999; Waddell et al. 2006; Goode et al. 2015), but less is known about its role in rapid re-acquisition after extinction.

The goal of the following experiments was to characterize the effects of several behavioral variables that may influence postextinction reconditioning of contextual fear (number and duration of extinction sessions, nonreinforced exposure to the context, a history of unconditioned stimulus (US) presentations, and sensitization in reconditioning) and to begin to characterize the brain regions involved by evaluating the effects of initial conditioning and postextinction reconditioning on histone acetylation and the expression of the immediate-early gene *c-Fos*, two markers of activity that have been shown to be involved in initial conditioning and extinction.

Results

Experiment 1: reconditioning after 2 or 12 extinction sessions

Because studies have found both rapid (Napier et al. 1992; Leung et al. 2007) and slow (Bouton 1986; Bouton et al. 2004; Leung et al. 2007) reacquisition following extinction, we evaluated the effects of different amounts of extinction on rapid reconditioning in Experiment 1. Two groups received unsignaled contextual fear conditioning followed by either moderate (2 sessions; Group REC 2 EXT) or extensive (12 sessions; Group REC 12 EXT) extinction. Those two groups and the third group with no history in the context (Group COND) then received a single weak conditioning session, followed by tests 1 and 4 d later.

Initial conditioning and extinction

An overview of the design of Experiment 1 is shown in Figure 1A. Freezing increased over the two acquisition sessions with no group differences ($F_{(1,7)}=26.7$, $P<0.01$; data not shown; session one: $M=41.03$, $SEM=5.76$, session two: $M=71.07$, $SEM=7.56$). There were no reliable main effects of or interactions with subsequent treatments during extinction or reconditioning in this or any subsequent experiment. Figure 1B shows the extinction curves for Groups REC 12 EXT and REC 2 EXT in 6-min time blocks for each session. Separate repeated-measures ANOVAs with session and time block as factors found reliable main effects of extinction session (P 's <0.002), time block (P 's <0.001), and a reliable interaction (P 's <0.05) in both groups. During the final half of the last extinction session, groups showed equivalently low levels of freezing (Extinction Session 12/2 in Fig. 1B), which showed that the two groups reached a common level of performance prior to reconditioning. All groups increased freezing after the shock in the reconditioning session (reliable main effect of Time [$F_{(1,21)}=35.67$, $P<0.001$; preshock: $M=1.04$, $SEM=1.04$, postshock: $M=42.71$, $SEM=7.13$]).

A

Experiment 1	Acquisition Days 1-2	Extinction Days 3-10	Extinction/ Acquisition Days 11-12	Extinction Days 13-14	Recond/ Cond Day 15	Tests Days 16, 19
REC 12 EXT	12 min + + + +	24 min -	24 min -	24 min -	3 min +	24 min-
REC 2 EXT	Handled		12 min + + + +	24 min -		
COND	Handled					

B

C

Figure 1. Rapid reacquisition occurs following 2 or 12 sessions of extinction. (A) Overview of the design of Experiment 1. The times listed represent the total time of exposure to the context for a given session. A “+” sign indicates a single 0.75 mA shock and a “-” sign indicates the exposure to the context without shock. (B) Mean freezing during each extinction session (E1–12 or E1–2) in rats that received fear conditioning Days 1 and 2 (REC 12 Ext) or Days 11 and 12 (REC 2 EXT). (C) Mean freezing during Tests 1 and 2 for each group. Tick marks on the X-axis reflect 6-min time blocks. (*) REC 12 EXT vs. COND, $P<0.05$; (#) REC 2 EXT vs. COND, $P<0.05$, and combined REC groups vs. COND, $P<0.01$. Error bars represent standard error of the mean.

Postreconditioning tests

All three groups were exposed to the context with no shock over two test sessions (Fig. 1C). Over the course of testing, the reconditioning groups did not differ as a function of the number of extinction sessions and showed more freezing early in the test sessions compared to the conditioning group. A 2 (Test Session) $\times$ 3 (Group) ANOVA revealed a reliable main effect of test session ($F_{(1,21)}=33.25$, $P<0.001$) and Time Block ($F_{(3,63)}=49.86$, $P<0.001$), as well as reliable interactions between Time Block and Group ($F_{(6,63)}=3.66$, $P=0.004$) and Test and Time Block ($F_{(3,63)}=4.67$, $P<0.01$). The interactions with Time Block were driven by different effects in the early blocks compared to the later blocks when freezing was lowest. Further analyses of the first 6 min of testing revealed that across the two tests, the two reconditioning groups did not differ from each other (P 's >0.40), but each reconditioning group differed from Group COND during one of the tests (Test 1: Group REC 12 EXT vs. COND, $q=2.54$, $P=0.048$; Test 2: Group REC 2 EXT vs. COND, $q=2.74$, $P=0.031$). When combined, the two reconditioning groups differed from the conditioning group over both tests ($t_{(22)}=2.949$, $P=0.007$). These results suggest that reconditioning after extinction is rapid and does not differ as a function of the number of extinction sessions.

Experiment 2: Effects of a postextinction US in a different context

The first experiment found that a single context-footshock pairing after extinction caused freezing to be reestablished in the context. It is conceivable that the quick reemergence of conditioned freezing following a postextinction context-shock pairing could be due to reinstatement to the US alone (Rescorla and Heth 1975) or a generalized sensitization of freezing due to a history of repeated shock (e.g., Rau and Fanselow 2009). The next two experiments

examined these possibilities. In Experiment 2, two groups of rats were conditioned in Context A and received 3 (Group REC 3 EXT) or 6 (Group REC 6 EXT) extinction sessions in Context A, followed by a brief reconditioning session in Context A. A separate group received conditioning in Context A followed by 3 sessions of extinction in Context A, then received brief conditioning in Context B (Group SWITCH 3 EXT). This group served as a control for the nonassociative impact of the postextinction shock on test freezing. A fourth group received no initial conditioning or extinction and only received the single shock in Context A (Group COND). All groups were tested in Context A the day after reconditioning, followed by a second test in Context B the next day, then a final test back in Context A.

Conditioning, extinction, and reconditioning

An overview of the design of Experiment 2 is shown in Figure 2A. All three reconditioning groups increased freezing from Acquisition Session 1 ($M=46.63$, $SEM=2.45$) to Session 2 ($M=66.78$, $SEM=3.83$; main effect of acquisition session ($F_{(1,14)}=35.3$, $P<0.001$)). As can be seen in Figure 2B, all extinction groups showed a decrease in freezing over the course of extinction. A two-way RMANOVA comparing REC 3 EXT and SWITCH 3 EXT across extinction showed a significant effect of Extinction session ($F_{(2,28)}=53.42$, $P<0.001$), and a separate one-way ANOVA found a reliable main effect of extinction session in REC 6 EXT ($F_{(5,35)}=38.12$, $P<0.001$). There were no differences in freezing among groups during the reconditioning session (data not shown). A RMANOVA comparing the average freezing 30 sec pre- and postshock (Time) across all groups in the reconditioning session found a significant main effect of Time ($F_{(3,28)}=36.05$, $P<0.001$; preshock: $M=0.00$,

$SEM=0.00$; postshock: $M=33.59$, $SEM=5.45$), but not Group ($F_{(3,28)}=0.487$, $P=0.694$) or Time $\times$ Group interaction ($F_{(3,28)}=0.487$, $P=0.694$).

Postreconditioning tests

Test 1: postreconditioning test in Context A. Twenty-four hours following weak reconditioning, all four groups were exposed to the original context (Context A) for 24 min (Fig. 2C). All groups that received the single footshock in Context A showed moderate to high levels of freezing at the beginning of Test 1, whereas Group SWITCH 3 EXT, which was shocked in Context B, showed no freezing in Context A. A Group $\times$ Test Block ANOVA revealed reliable main effects of Group and Test Block (F 's > 10.9 , P 's < 0.001), as well as a reliable interaction ($F_{(9,84)}=4.40$, $P<0.001$). The two reconditioning groups did not differ from each other ($P=0.331$) and both differed from Group SWITCH 3 EXT (P 's < 0.005). Group REC 6 EXT differed from the Group COND ($P=0.021$), but Group REC 3 EXT was not reliably different from Group COND.

Test 2: postreconditioning test in Context B. When tested in Context B, only Group SWITCH 3 EXT showed robust freezing (Fig. 2C, Context B), which was the context in which they were previously shocked. A Group $\times$ Test Block ANOVA revealed reliable main effects of Test Block and Group ($F_{(3,84)}=16.85$ and $F_{(3,28)}=8.269$, respectively, P 's < 0.001), as well as a reliable interaction ($F_{(9,84)}=10.03$, $P<0.001$). Follow-up Tukey tests found that Group SWITCH 3 EXT differed from each of the other three groups over the session (P 's < 0.005). This result showed that acquisition and rapid reacquisition did not generalize across contexts, as Groups COND, REC 3 EXT, and REC 6 EXT showed little freezing in a different context.

Test 3: second test in Context A. The final test consisted of a reexposure to Context A (Fig. 2C). A Group $\times$ Test Block ANOVA revealed reliable main effects of Test Block and Group ($F_{(1,27)}=33.339$ and $F_{(3,27)}=4.123$, P 's < 0.02), as well as a reliable interaction ($F_{(3,27)}=6.335$, $P=0.002$). Follow-up Tukey's HSD tests found that both reconditioning groups differed from the conditioning group during the first 6 min of Test 3 (REC 6 EXT vs. COND, $P=0.004$; REC 3 EXT vs. COND, $P=0.04$). It is notable that Group COND did not freeze during this test, suggesting that extinction in the first two tests eliminated freezing in the COND group, but not in the REC groups.

These findings show that the reconditioning effect is specific to the context in which reconditioning occurs. The absence of freezing in Context A in the SWITCH 3 EXT group shows that the freezing observed in the reconditioning groups is not due simply to generalization or sensitization. This experiment also replicated the rapid reconditioning effect observed in Experiment 1, but, in Test 1, this effect only occurred in the reconditioning group that received more extinction; no difference was observed between the moderate extinction group and the conditioning group. However, the freezing that was established in Group COND rapidly extinguished and did not persist to a second test in Context A, whereas the

Figure 2. Rapid reacquisition of contextual fear occurs after 3 or 6 sessions of extinction and is specific to the context of reconditioning. (A) Overview of the design of Experiment 2. The times listed represent the total time of exposure to the context for a given session. A "+" sign indicates a single 0.75 mA shock and a "-" sign indicates the exposure to the context without shock. The treatment context is represented by A or B. (B) Mean freezing during each extinction session (E1–6 or E1–3) in rats that received fear conditioning Days 1 and 2 (REC 6 EXT) or Days 4 and 5 (REC 3 EXT and REC 3 SWITCH). (C) Mean freezing during the three tests for each group. Tick marks on the X-axis reflect 6-min time blocks. (*) REC groups vs. SWITCH, $P<0.005$; REC 6 EXT vs. COND, $P<0.05$; (#) SWITCH vs. all other groups, $P<0.005$; (^) REC groups vs. COND, P 's < 0.05 . Error bars represent the standard error of the mean.

freezing in both reconditioning groups did. Thus, reconditioning effects may be manifest early or late in testing.

Experiment 3: reconditioning with matched US and context exposure

In Experiment 3, we again evaluated the effects of exposure to shock on nonassociative performance, as well as the potential contribution of differences in context exposure as a contributor to the differences between conditioning and reconditioning groups. In Experiments 1 and 2, the reconditioning groups received more total context exposure than did the conditioning group. This is a potential confound that could weaken reconditioning (through latent inhibition effects; e.g., Mowrer 1987) or strengthen reconditioning (through perceptual learning and/or conjunctive representational effects; e.g., Rudy and O'Reilly 1999). In this experiment, four groups received conditioning with a single shock in Context A. For Group REC SAME, this occurred after initial conditioning and extinction in Context A; for Group REC DIFF, this occurred after an initial conditioning and extinction in Context B; for Group COND SAME, this occurred after equivalent nonreinforced exposure to Context A; for Group COND DIFF, this occurred after an identical amount of nonreinforced exposure to Context B. Thus, Group REC SAME received the single shock after conditioning and extinction, as in Experiments 1 and 2. Group REC DIFF served as a control to match the history of shock exposure and nonreinforced context exposure. Group COND SAME served as a control that received identical context exposure to address potential latent inhibition or perceptual learning. Group COND DIFF was the same group used in Experiments 1 and 2, but it received nonreinforced exposure to Context B to match handling and context exposure to Group COND SAME.

Results

Conditioning, extinction, and reconditioning

An overview of the design of Experiment 3 is shown in Figure 3A. Shocked groups increased freezing from Acquisition Session 1 ($M = 50.74$, $SEM = 3.63$) to Session 2 ($M = 78.91$, $SEM = 3.15$), whereas No Shock groups did not freeze ($M < 1.0\%$ in each session); main effects of Session ($F_{(1,30)} = 98.04$, $P < 0.001$), Shock Group ($F_{(1,30)} = 434.2$, $P < 0.001$) and a reliable interaction ($F_{(1,30)} = 102.03$, $P < 0.001$). As can be seen in Figure 3B, the shocked groups showed a decrease in freezing over the course of extinction, while the non-shocked groups continued to maintain low levels of freezing. An ANOVA found reliable main effects of extinction session, time block, and group (all P 's < 0.001).

Test

Figure 3C shows freezing during the 24-min test in Context A. Both reconditioning groups showed higher levels of freezing compared to the conditioning groups and the two reconditioning groups differed from each other. A 2 (Context of postextinction shock: Same or Different) $\times$ 2 (Conditioning: Initial Conditioning or Reconditioning) $\times$ 4 (Test Block) ANOVA revealed reliable main ef-

Figure 3. Effects of shock history and context exposure on conditioning and reconditioning. (A) Overview of the design of Experiment 3. The times listed represent the total time of exposure to the context for a given session. A “+” sign indicates a single 0.75 mA shock and a “-” sign indicates the exposure to the context without shock. (B) Mean percent freezing during extinction or context pre-exposure. (C) Mean percent freezing during the test. Tick marks on the X-axis reflect 6-min time blocks. (*) REC SAME vs. REC DIFF, $P < 0.05$. Error bars represent the standard error of the mean.

fects of Test Block ($F_{(3,84)} = 14.30$, $P < 0.001$), Conditioning ($F_{(1,28)} = 16.63$, $P < 0.001$), and Context ($F_{(1,28)} = 5.00$, $P = 0.033$), as well as reliable interactions between Time Block and Conditioning ($F_{(3,84)} = 8.36$, $P < 0.001$) and Conditioning and Context ($F_{(1,28)} = 7.03$, $P = 0.013$). Further analysis of the interactions found more test freezing in Group REC SAME compared to Group REC DIFF ($P = 0.02$) and that both reconditioning groups differed from both conditioning groups (P 's < 0.005), which did not differ from each other. These findings confirm the findings from Experiment 2 that reconditioning effects are not due entirely to nonassociative contributions of the postextinction shock. However, a history of conditioning did result in the single footshock having a much larger impact compared to groups that did not have that history, consistent with findings in the stress-enhanced fear learning literature (SEFL) (Rau et al. 2005). Finally, this experiment found no evidence that context preexposure promoted conditioning with the single footshock and appeared to weaken responding in a context-independent manner, possibly suggesting that extensive handling and context exposure weakens the impact of a single shock.

Experiment 4: reconditioning after short or long extinction sessions

The first three experiments found rapid reacquisition after extinction. This effect was not always robust (e.g., Group REC 3 EXT in Experiment 2), but there was no evidence that postextinction reconditioning was impaired relative to initial conditioning. There are many demonstrations that massive extinction may slow reacquisition (Bouton 1986; Leung et al. 2007) and we have previously found that massive extinction impairs rapid reacquisition of contextual fear in mice (Williams and Lattal 2019). It is notable that in Experiments 1–3 freezing extinguished quickly and showed little spontaneous recovery across extinction sessions. Some work has shown that preventing conditioned responding during extinction can impair extinction learning (Krupa and Thompson 2003;

Sierra-Mercado et al. 2006) and the absence of spontaneous recovery may create a less persistent extinction effect (Leung and Westbrook 2008). Further, a lack of conditioned responding in later extinction sessions could be preventing prediction error mechanisms that allow for extinction (Rescorla 2000) or gradual extinction that could cause stronger inhibition of conditioned responding (Gershman et al. 2013). All of this would suggest that our extinction procedures with large numbers of extinction sessions (6 or 12) did not have much additional impact once responding had ceased. Thus, in Experiment 4 we examined if more gradual suppression of conditioned freezing over multiple extinction sessions could deepen inhibitory learning and impair rapid reacquisition.

Results

Conditioning, extinction, and reconditioning

The design of Experiment 4 is summarized in Figure 4A. All groups increased freezing across the two acquisition sessions ($F_{(1,20)} = 36.149$, $P < 0.001$; session one: $M = 39.97$, $SEM = 4.24$; session two: $M = 65.21$, $SEM = 4.32$). Figure 4B shows extinction in 3-min time blocks. Extinction caused conditioned freezing to lower in both groups. To compare the two extinction durations during the course of extinction, we compared groups during the first 6 min of each session. A RMANOVA found a significant main effect of Group ($F_{(1,20)} = 4.89$, $P < 0.05$) and Extinction session ($F_{(3,60)} = 104.67$, $P < 0.001$), as well as a significant interaction ($F_{(3,60)} = 3.95$, $P = 0.012$). Further analysis of the interaction found no group differences in the first 6 min of Extinction Sessions 1 or 4 (P 's > 0.21), but there were differences in Extinction Sessions 2 and 3 (P 's < 0.05). It is worth noting that one animal in the 6-min group stayed at 100% freezing during the course of extinction and testing, which kept the group means above zero during the final extinction block. Median percent freezing during the final 3-min of the last extinction session was 0.0 in both groups. Thus, the two groups began and ended extinction with similar levels of freezing,

meaning that both groups started reconditioning from a common low level of performance.

During reconditioning, groups did not show within-session differences in freezing (data not shown). A RMANOVA comparing the freezing 30 sec pre- and postshock found a significant effect of Time ($F_{(1,20)} = 12.36$, $P < 0.005$; preshock: $M = 6.81$, $SEM = 4.70$, postshock: $M = 32.95$, $SEM = 8.61$), but not a significant main effect of Group ($F_{(1,20)} = 0.74$, $P = 0.400$) or a Time $\times$ Group Interaction ($F_{(1,20)} = 0.023$, $P = 0.880$).

Test 1: postreconditioning test

Figure 4C shows freezing during the postreconditioning test. A RMANOVA found no reliable main effect of Group ($F_{(1,20)} = 1.47$, $P = 0.239$), but did find a reliable main effect of Time Block ($F_{(7,140)} = 6.682$, $P < 0.001$) and a reliable Time Block $\times$ Group interaction ($F_{(7,140)} = 2.110$, $P = 0.046$). The most liberal simple effects analysis revealed a group difference only on Block 2 ($t_{(20)} = 2.36$, $P = 0.028$). This finding suggests that, as with the number of extinction sessions, duration of extinction session has little impact on the amount of reconditioning.

Experiment 5: immunohistochemical characterization of rapid reconditioning

These first four experiments demonstrated that postextinction reconditioning is associative, rapid, and robust against different extinction treatments; it occurs at similar levels when different number or durations of extinction sessions are used. To begin to explore the brain mechanisms underlying reconditioning, we conducted an immunohistochemical experiment to examine different brain regions that may be engaged by postextinction reconditioning. We focused on histone acetylation and c-Fos expression in the mPFC, amygdala, hippocampus, and BNST, regions that have been shown to be involved in fear conditioning and extinction. By using the same conditioning procedure (a single context-shock pairing) at the same time for each group, this experiment allowed for the direct comparison of acquisition and reacquisition

on the brain regions important for fear learning. Three groups received a single context-shock pairing prior to sacrifice. For Group COND, this was their first conditioning episode (initial conditioning); for Groups REC-EXT and REC-NO EXT, this was their second conditioning episode (reconditioning). Group REC-EXT received extinction between conditioning and reconditioning; Group REC-NO EXT did not receive extinction prior to reconditioning. Group HAND was only handled. These groups allowed us to compare initial conditioning with postextinction reconditioning, and the inclusion of REC-NO EXT allowed us to evaluate the effects of the second conditioning episode independent of extinction.

Conditioning, extinction, and reconditioning

The design of Experiment 5 is summarized in Figure 5A. Over two contextual fear conditioning sessions, both REC-EXT and REC-NO EXT acquired conditioned freezing to the context. A two-way RMANOVA comparing Group $\times$ Acquisition Session showed a significant within-subjects effect of Acquisition

Figure 4. Reacquisition does not differ as a function of the extinction session duration. (A) Overview of the design of Experiment 4. The times listed represent the total time of exposure to the context for a given session. A "+" sign indicates a single 0.75 mA shock and a "-" sign indicates the exposure to the context without shock. (B) Mean freezing during each extinction session. (C) Mean freezing during the test for each group. Tick marks on the X-axis reflect 3-min time blocks. Error bars represent standard error of the mean.

Figure 5. Extinction and rapid reacquisition prior to immunohistochemistry. (A) Overview of the design of Experiment 4. The times listed represent the total time of exposure to the context for a given session. A “+” sign indicates a single 0.75 mA shock and a “-” sign indicates the exposure to the context without shock. (B) The acquisition and extinction curves from Groups REC-EXT and REC-NO EXT as shown by the mean percent freezing for each session; A = acquisition session, E = extinction session (C) Mean percent freezing in the 30 sec pre- and postshock on Day 6 during reconditioning. Rapid reacquisition of freezing relative to initial acquisition occurred in both REC groups, but only in REC-EXT relative to preshock freezing. Significance between groups is represented by (***) $P < 0.001$; (*) $P < 0.05$. REC-EXT, $n = 6$; REC-NO EXT, $n = 6$; COND, $n = 6$; HAND, $n = 6$. Error bars represent standard error of the mean.

Session ($F_{(1,10)} = 36.86$, $P < 0.001$; session one: $M = 61.24$, $SEM = 2.44$, session two: $M = 83.20$, $SEM = 3.92$), but not a main effect of Group ($F_{(1,10)} = 0.32$, $P = 0.583$; REC-EXT: $M = 73.83$, $SEM = 4.76$, REC-NO EXT: $M = 70.61$, $SEM = 4.49$) or an interaction ($F_{(1,10)} = 0.386$, $P = 0.548$). During 3 sessions of extinction, animals in REC-EXT decreased their conditioned freezing response (Fig. 5B). A one-way RMANOVA revealed a significant within-subjects effect of Extinction Session on session average percent freezing ($F_{(2,10)} = 16.69$, $P < 0.001$) that was driven by the significantly lower freezing in E3 compared to E1 ($q_{(15)} = 3.7948$, $P < .05$). While E3 session average freezing did not show animals extinguishing to 0% freezing, all animals in REC-EXT were near 0% freezing in the last 3 min of E3 ($M = 2.89$, $SEM = 2.89$).

During reconditioning, the reconditioning group that had not received extinction (REC-NO EXT) showed high levels of freezing before and after the shock. Groups REC-EXT and COND showed low levels of freezing prior to the shock and increased by different amounts after the shock (Fig. 5C). A RMANOVA comparing percent time spent freezing in the 30 sec before and aftershock by each Group (Time $\times$ Group) found a significant main effect of Group ($F_{(2,15)} = 23.46$, $P < 0.001$), within-subjects effect of Time ($F_{(1,15)} = 34.41$, $P < 0.001$), and a trending interaction effect ($F_{(2,15)} = 3.55$, $P = 0.054$). The main effect of Time was caused by significantly more freezing shown aftershock relative to before shock and the main effect of Group was driven by significantly more freezing by REC-NO EXT relative to COND ($q_{(15)} = 7.10$, $P < 0.001$) and REC-EXT ($q_{(15)} = 4.44$, $P < 0.001$). Additionally, REC-EXT showed significantly more freezing than COND ($q_{(15)} = 2.65$, $P < 0.05$), indicating that both reconditioning groups froze more than COND and that a lack of extinction training causes

higher freezing in general (REC-NO EXT $>$ REC-EXT). The trending interaction effect showed that only REC-EXT had significantly more freezing postshock relative to preshock ($q_{(15)} = 6.85$, $P < 0.001$), while COND ($q_{(15)} = 1.07$, $P = 0.262$) or REC-NO EXT did not ($q_{(15)} = 0.671$, $P = 0.957$). Animals were euthanized 50–90 min after reconditioning for immunohistochemistry.

c-Fos immunohistochemistry

All regions and subregions were analyzed for group differences in c-Fos-positive cells. Representative DAB IHC staining for c-Fos for each group in a significant region (DG) can be seen in Figure 6A. The full summary of results is in Table 1.

Only the hippocampus had a significant difference in c-Fos among groups in whole region analyses (Table 1). A one-way ANOVA found a significant main effect of Group ($F_{(3,19)} = 11.1$, $P < 0.001$) that was driven by significantly more c-Fos-positive cells in COND ($q_{(19)} = 7.95$, $P < 0.001$), REC-NO-EXT ($q_{(19)} = 5.40$, $P < 0.01$), and REC-EXT ($q_{(19)} = 4.84$, $P < .05$) relative to HAND. Subsequent analyses focused on the DG (Table 1; Fig. 7). In a one-way ANOVA comparing c-Fos-positive cells in the DG among groups there was a significant main effect of Group ($F_{(3,19)} = 16.6$, $P < 0.001$), driven by significantly more DG c-Fos expression in REC-EXT ($q_{(19)} = 4.43$, $P < 0.05$),

REC-NO EXT ($q_{(19)} = 5.68$, $P < 0.01$), and COND ($q_{(19)} = 9.93$, $P < 0.01$) relative to HAND and significantly more DG c-Fos expression in COND relative to REC-EXT ($q_{(19)} = 5.04$, $P < 0.05$) and REC-NO EXT ($q_{(19)} = 4.25$, $P < 0.05$). This effect suggests that both acquisition and reacquisition cause increased DG c-Fos expression, but that initial acquisition causes the strongest activation of DG c-Fos expression.

While the aBNST as a whole did not show significant group differences in c-Fos expression, there was a reliable main effect of Group in the adBNST subregion ($F_{(3,20)} = 6.16$, $P < 0.01$) due to significantly more c-Fos-positive cells in COND relative to HAND ($q_{(20)} = 5.66$, $P < 0.01$) and REC-EXT ($q_{(20)} = 4.76$, $P < 0.05$). Similar to the DG, this result implies that initial acquisition causes a stronger activation of c-Fos in the adBNST relative to reacquisition or simple handling.

H4K8ac immunohistochemistry

All regions and subregions were analyzed for group differences in H4K8ac-positive cells, but only the lateral amygdala subregion showed significant differences. Representative fluorescent IHC staining for H4K8ac for each group in a significant region (LA) can be seen in Figure 6B. A summary of the results of each region is in Table 2. A one-way ANOVA found a significant main effect of Group on cells positive for H4K8ac within the lateral amygdala ($F_{(3,18)} = 3.64$, $P < 0.05$). The significant Group effect was driven by significantly more H4K8ac-positive cells in COND relative to HAND controls ($q_{(18)} = 4.48$, $P < 0.05$), which suggested that conditioning significantly activated histone acetylation within the lateral portion of the amygdala (Fig. 7D).

Figure 6. Representative coronal slices of c-Fos and H4K8ac staining. (A) The four panels are representative images of average c-Fos staining in DG for each group that resulted from DAB IHC. The large-dashed white line represents what region was counted for the DG. The small-dashed black square shows a magnified region of the slices with white arrows indicating cells showing positive DAB staining (not all stained cells are indicated with an arrow to maintain clarity). (B) The four panels are representative images of average H4K8ac staining in LA for each group that resulted from fluorescent IHC. The large-dashed white line represents what region was counted for the LA. The small-dashed white square shows a magnified region of the slices with white arrows indicating cells showing positive fluorescent staining (not all stained cells are indicated with an arrow to maintain clarity).

General discussion

These experiments demonstrated that rapid reacquisition of contextual fear occurs following extinction in male Long-Evans rats. Thus, as with other postextinction procedures, rapid reconditioning of contextual fear appears to involve the unmasking of a context-shock association that is suppressed by extinction. This ef-

fect was for the most part independent of the amount of extinction and returned after the postreconditioning behavior had been extinguished during initial tests. Additionally, this work shows that rapid reacquisition of contextual fear is not a result of (1) a generalized enhancement in fear that may occur after reexposure to a shock US in a different context, (2) a generalized increase in responsivity to a single US following extensive conditioning in a different context (although this did cause some generalized increase in freezing), or (3) increased contextual learning relative to conditioning groups following nonreinforced context exposure. Thus, the return of postextinction freezing is a specific context-associated effect of reconditioning. Finally, although reconditioning increased histone acetylation or c-Fos expression relative to handling controls, this effect was not as large as the effect of initial conditioning.

The finding of rapid reacquisition following extinction has been seen many times in the literature (e.g., Napier et al. 1992; Bouton et al. 2004; Leung et al. 2007) and is consistent with other postextinction processes, such as spontaneous recovery, reinstatement, and contextual renewal (Pavlov 1927; Rescorla and Heth 1975; Bouton and Bolles 1979a,b; Robbins 1990; Rescorla 2004). The current findings reinforce reacquisition as yet another way by which to study the way in which fear promoting and inhibitory associative memories interact and compete for expression during and after extinction.

Although some studies have found that extensive extinction weakens postextinction reconditioning, sometimes to the point that reacquisition is slower compared to the initial acquisition, we found that extensive extinction had no effect on reconditioning. This is in contrast to our previous work with mice, which found that extensive extinction eliminated rapid reconditioning (Williams and Lattal 2019). The slow rate of reacquisition that sometimes occurs could be due to a number of factors, including performance variables and differences in exposure to the context causing latent inhibition (Bouton and Swartzentruber 1989; Brooks et al. 2003; Williams and Lattal 2019). We found that extensive context preexposure did appear to weaken initial conditioning, but this was not

specific to the context of preexposure (Experiment 3). Comparing our results with rats with our previous work in mice is complicated because conditioned freezing in mice is difficult to distinguish from general inactivity. This may mask any subtle reconditioning effects because nonassociative freezing in mice is above zero and extinction does not reach a level of zero percent freezing (Lattal and Maughan 2012; Tipps et al. 2014; Crabbe et al. 2016; Williams

Table 1. Mean $\pm$ SEM of c-Fos-positive cells in every region selected following Day 6

	REC-EXT	REC-NO EXT	COND	HAND	ANOVA RESULT
<i>mPFC</i>	294.3 $\pm$ 53.3	260.4 $\pm$ 26.9	298.6 $\pm$ 39.8	206.1 $\pm$ 34.5	$F_{(3,20)} = 1.15, P = 0.352$
<i>IL</i>	124.0 $\pm$ 20.3	117.3 $\pm$ 11.6	131.6 $\pm$ 18.8	82.1 $\pm$ 13.9	$F_{(3,20)} = 1.75, P = 0.188$
<i>PrL</i>	170.3 $\pm$ 33.8	143.1 $\pm$ 18.3	167.1 $\pm$ 23.7	124.0 $\pm$ 20.8	$F_{(3,20)} = 0.78, P = 0.519$
<i>Amygdala</i>					
<i>LA</i>	14.6 $\pm$ 3.8	13.9 $\pm$ 2.6	23.4 $\pm$ 3.6	17.3 $\pm$ 3.0	$F_{(3,20)} = 1.73, P = 0.194$
<i>BLA</i>	32.3 $\pm$ 6.5	23.9 $\pm$ 5.4	35.8 $\pm$ 5.5	41.5 $\pm$ 5.9	$F_{(3,20)} = 1.60, P = 0.220$
<i>CeA</i>	6.2 $\pm$ 2.9	7.2 $\pm$ 1.6	12.5 $\pm$ 3.0	5.5 $\pm$ 1.0	$F_{(3,20)} = 1.94, P = 0.155$
<i>dHipp</i>	22.0 $\pm$ 2.8^a (<i>n</i> = 5)	23.0 $\pm$ 3.5^a	30.0 $\pm$ 3.2^a	8.0 $\pm$ 1.5	$F_{(3,19)} = 11.1, P < 0.001$
<i>CA1</i>	1.6 $\pm$.23 (<i>n</i> = 5)	1.3 $\pm$.42	0.71 $\pm$ 0.23	1.1 $\pm$ 0.5	$F_{(3,19)} = 0.913, P = 0.453$
<i>CA2</i>	0.38 $\pm$.19 (<i>n</i> = 5)	0.52 $\pm$.18	0.33 $\pm$ 0.19	0.06 $\pm$ 0.06	$F_{(3,19)} = 1.53, P = 0.238$
<i>CA3</i>	4.6 $\pm$ 1.4 (<i>n</i> = 5)	3.4 $\pm$ 1.2	1.8 $\pm$ 0.36	1.6 $\pm$ 0.41	$F_{(3,19)} = 2.22, P = 0.119$
<i>DG</i>	15.5 $\pm$ 2.4^{a,b} (<i>n</i> = 5)	17.7 $\pm$ 2.8^{a,b}	27.1 $\pm$ 2.5^a	5.2 $\pm$ 0.84	$F_{(3,19)} = 16.6, P < 0.001$
<i>aBNST</i>	84.0 $\pm$ 30.1	100.7 $\pm$ 18.6	160.1 $\pm$ 29.8	76.8 $\pm$ 11.4	$F_{(3,20)} = 2.52, P = 0.087$
<i>adBNST</i>	30.7 $\pm$ 10.3^b	42.22 $\pm$ 5.8	73.0 $\pm$ 12.9^a	22.7 $\pm$ 3.3	$F_{(3,20)} = 6.16, P < 0.01$
<i>avBNST</i>	53.3 $\pm$ 20.4	58.5 $\pm$ 13.2	87.1 $\pm$ 19.6	54.1 $\pm$ 8.3	$F_{(3,20)} = 0.98, P = 0.418$

^aSignificantly higher compared with HAND at $P < 0.05$.^bSignificantly less than COND at $P < 0.05$.*n* = 6, unless otherwise specified. Bold indicates significance between groups.

and Lattal 2019), in contrast to the relatively fast extinction to zero observed in rats in the present experiments and many others (Quirk et al. 2000; Anglada-Figueroa and Quirk 2005; Leung et al. 2007; Laurent and Westbrook 2008).

Although other studies have also found rapid reacquisition even after extensive extinction (e.g., Willcocks and McNally 2011), still others have found slow reacquisition after more extensive extinction (Bouton 1986; Bouton et al. 2004; Leung et al. 2007). It is possible that the rapid loss of conditioned responding during extinction in our experiments prevented extinction from strengthening. We observed very little spontaneous recovery after the first couple of extinction sessions, which may have prevented large prediction error-driven learning during the early parts of each subsequent extinction session, which is thought to be important for strengthening extinction (Wagner 1970; Rescorla 2000; Krupa and Thompson 2003; Sierra-Mercado et al. 2006; Leung and Westbrook 2008). However, it is notable that the second extinction session in Figures 1B and 2B appeared to result in weaker spontaneous recovery in groups receiving fewer extinction sessions compared to the more extensive extinction groups. It is not clear why this would occur, except that there may have been overall differences in the colony that could have contributed to lower levels of fear after one group had been extensively extinguished (Bredy and Barad 2008). It is hard to compare the second extinction session in the different groups because they occurred on different days to allow us to focus on common treatments during reconditioning and testing.

We attempted to manipulate the amount of spontaneous recovery that may occur by shortening the extinction session to allow for the more prolonged expression of the conditioned response in Experiment 4. Between-session differences in expression of fear did not affect reacquisition. Indeed, the literature is mixed in terms of the effects of expression of the conditioned response and extinction speed on the persistence of extinction, where expression and gradual extinction increases or does not affect extinction (e.g., Ouyang and Thomas 2005; Zimmerman and Maren 2010; Gershman et al. 2013).

It is also important to note that our initial conditioning procedure consisted of 2 d of conditioning, in which rats received four footshocks per day. Ricker and Bouton (1996) showed that conditioning procedures that require more conditioning sessions for acquisition favored rapid reacquisition following reconditioning due to sequential learning, which occurs when a CS–US pairing

predicts further CS–US pairings due to extensive acquisition training (Capaldi 1994). Thus, our use of two 4-shock conditioning sessions could favor rapid reacquisition and overcome the potentially impairing effect of massive extinction.

Additionally, the behavioral preparation here differed from a similarly conducted study that found that massive extinction impaired rapid reacquisition in Long–Evans rats (Leung et al. 2007). In the work by Leung et al. both conditioning and reconditioning sessions were a short 3 min context exposure with one shock (delivered 1 min into the session) and extinction was twice-daily with an extinction criterion requiring the absence of freezing across the initial minute of exposure. Perhaps procedural differences, such as weaker initial conditioning and twice-daily extinction sessions, led to changes in the strength of both conditioning and extinction memories that allowed rapid reacquisition to be impaired by massive extinction. The evidence of slow reacquisition found by the design in Leung et al. (2007) is relevant to the theory that additional trials during initial conditioning in Experiments 1 and 2 might have allowed sequential learning to favor rapid reacquisition in rats, regardless of the number of extinction sessions.

There are many other possible causes for rapid reacquisition. Similar to sequential learning, another possibility is that the shock becomes encoded as part of the acquisition context, meaning that a postextinction shock functionally serves to return the animals to the original context (Bouton 2002; Willcocks and McNally 2011), an account supported by the finding that intermittent CS–US pairings during extinction can slow reacquisition (Bouton et al. 2004) and presentation of outcomes associated with extinction can weaken resurgence (Trask and Bouton 2016). A related account would suggest that the shock delivered during reconditioning could have acted as a reminder of the original CS–US association and caused reinstatement of the fear behavior as opposed to an actual reconditioning of the CS–US association (Bouton and Bolles, 1979b). Likewise, rapid reacquisition could be a result of a generalized enhancement of freezing behavior due to repeated exposure to shock. Rodents may have become sensitized to shock and exposure to further shock in any context could enhance fear learning, as in SEFL (Rau and Fanselow 2009). In Experiment 2, we found that a postextinction shock in a different context (Context B) from the conditioning and extinction contexts (Context A) did not cause increased freezing in the extinction context (Context A), but in Experiment 3 we found that this treatment did cause a generalized

Figure 7. Acquisition leads to stronger activity marker expression than rapid reacquisition. (A) The bar graph on the *left* shows the average c-Fos-positive cells within the entire dorsal hippocampus for each group. The image on the *right* shows a representative coronal slice of the hippocampus and the solid white line shows the region that was assessed for creating the average c-Fos-positive cell counts for analysis. (B) A similar figure for the DG of the hippocampus. (C) A similar figure for the adBNST. (D) The bar graph on the *left* shows the average H4K8ac-positive cells within the lateral amygdala (LA) for each group. The panel on the *right* shows a representative coronal slice of the LA and the solid white line shows the region that was assessed for creating the average H4K8ac-positive cells. Significance between groups is represented by (***) $P < 0.001$; (**) $P < 0.01$; (*) $P < 0.05$. Group numbers for each region are detailed in Tables 1, 2. Error bars represent standard error of the mean.

Table 2. Mean $\pm$ SEM of H4K8ac-positive cells in every region selected following Day 6

	REC-EXT	REC-NO EXT	COND	HAND	ANOVA RESULTS
<i>mPFC</i>	273.6 $\pm$ 80.1	510.3 $\pm$ 233.8, (<i>n</i> = 5)	645.8 $\pm$ 253.4	198.5 $\pm$ 49.0, (<i>n</i> = 4)	$F_{(3,17)} = 0.788$, $P = 0.517$
<i>IL</i>	264.5 $\pm$ 116.5	157.1 $\pm$ 33.8	324.5 $\pm$ 122.7	115.2 $\pm$ 33.3, (<i>n</i> = 4)	$F_{(3,18)} = 0.958$, $P = 0.434$
<i>PrL</i>	245.8 $\pm$ 117.5	153.3 $\pm$ 42.3 (<i>n</i> = 5)	285.2 $\pm$ 96.3	121.8 $\pm$ 34.5, (<i>n</i> = 4)	$F_{(3,17)} = 0.677$, $P = 0.578$
<i>Amygdala</i>					
LA	136.1 $\pm$ 45.5	121.8 $\pm$ 38.3 , (<i>n</i> = 4)	261.3 $\pm$ 57.3^a	77.2 $\pm$ 11.4	$F_{(3,18)} = 3.64$, $P < 0.05$
<i>BLA</i>	146.8 $\pm$ 56.4	63.8 $\pm$ 15.4, (<i>n</i> = 4)	181.6 $\pm$ 46.2	81.42 $\pm$ 20.8	$F_{(3,18)} = 1.66$, $P = 0.212$
<i>dHipp</i>	52.3 $\pm$ 20.6 (<i>n</i> = 5)	24.0 $\pm$ 13.6 (<i>n</i> = 5)	66.1 $\pm$ 40.0	12.1 $\pm$ 4.3	$F_{(3,18)} = 1.06$, $P = 0.390$
<i>CA1</i>	16.6 $\pm$ 9.9 (<i>n</i> = 5)	2.7 $\pm$ 1.6	23.7 $\pm$ 9.7	2.1 $\pm$ 0.7	$F_{(3,19)} = 1.74$, $P = 0.193$
<i>CA2</i>	3.0 $\pm$ 1.3 (<i>n</i> = 5)	1.8 $\pm$ 1.2 (<i>n</i> = 5)	4.5 $\pm$ 2.6	0.8 $\pm$ 0.3	$F_{(3,18)} = 0.99$, $P = 0.419$
<i>CA3</i>	2.3 $\pm$.2 (<i>n</i> = 5)	1.7 $\pm$.7 (<i>n</i> = 5)	2.9 $\pm$ 1.1	1.2 $\pm$ 0.5	$F_{(3,18)} = 1.23$, $P = 0.328$
<i>DG</i>	30.4 $\pm$ 10.6 (<i>n</i> = 5)	15.0 $\pm$ 8.8	40.4 $\pm$ 26.9	8.1 $\pm$ 3.4	$F_{(3,19)} = 0.90$, $P = 0.459$
<i>aBNST</i>	530.0 $\pm$ 116.3	405.6 $\pm$ 39.1	610.8 $\pm$ 162.4	479.4 $\pm$ 45.5	$F_{(3,20)} = 0.685$, $P = 0.572$
<i>adBNST</i>	227.8 $\pm$ 52.5	185.4 $\pm$ 19.8	283.4 $\pm$ 84.3	191.3 $\pm$ 22.2	$F_{(3,20)} = 0.756$, $P = 0.532$
<i>avBNST</i>	302.2 $\pm$ 69.0	220.2 $\pm$ 26.0	327.3 $\pm$ 83.2	288.1 $\pm$ 30.7	$F_{(3,20)} = 0.631$, $P = 0.603$

^aSignificantly higher compared with HAND at $P < 0.05$.*n* = 6, unless otherwise specified. Bold indicates group differences.

increase in freezing in Context B, relative to groups receiving conditioning in Context B for the first time (i.e., a SEFL effect). Nonetheless, in both cases, the impact of the postextinction shock was the largest in the group that had received conditioning, extinction, and reconditioning all in the same context. This suggests that reconditioning occurs through associative processes that are not entirely due to sensitization or reinstatement from exposure to a postextinction shock.

Our experiments did not directly address some additional theoretical causes of rapid reacquisition: sequential learning, learning-to-learn, or the ability of rapid reacquisition to reactivate and strengthen the original excitatory memory. Yet, these experiments show that rapid reacquisition cannot be conflated with reinstatement to the US nor can it be considered a general enhancement in freezing to any context. Rapid reacquisition of contextual fear is likely its own unique phenomenon caused by CS-specific learning. Given these multiple accounts and other demonstrations that reconditioning and other postextinction processes differ at the behavioral level (e.g., Bouton et al. 2012; Willcocks and McNally 2014), it will be important to continue to characterize the behavioral effects of reconditioning.

At a neurobiological level, our immunohistochemistry results did not reveal that any of the structures we examined were specifically activated by postextinction reconditioning. When we did observe reliable group differences, the consistent finding was that initial conditioning resulted in more c-Fos expression and histone acetylation compared to handling controls. However, in some cases, reconditioning also elevated c-Fos expression above handling controls.

We found that the lateral subregion of the amygdala had greater H4K8 acetylation following weak initial acquisition than rapid reacquisition following extinction, reacquisition without extinction, and no-learning controls. An increase of H4K8ac following only acquisition suggests that this region was important for the molecular processes during and leading to the acquisition of a contextual fear memory. The lateral amygdala is a region known for being the entryway for sensory information that will be part of an associative memory (LeDoux et al. 1990; Maren and Quirk, 2004) and is necessary for cued and contextual fear conditioning (Goosens and Maren 2001), as well as extinction (e.g., Hobin et al. 2003; Laurent et al. 2008; Stafford et al. 2013). Particularly relevant to this finding, epigenetic processes, including histone acetylation, within the LA were critical for synaptic plasticity during and consolidation of fear (Monsey et al. 2011; Maddox et al. 2013; Kwapis et al. 2017). Given these findings and the LA's role

as the sensory gateway for associative memories, the LA was likely to increase H4K8ac expression immediately following exposure of context-shock pairing.

We also found that the dorsal hippocampus, particularly the dentate gyrus region, was activated by conditioning and reconditioning. Many studies have shown the importance of the dorsal hippocampus in contextual fear conditioning and extinction (Phillips and LeDoux 1992; Lee and Kesner 2004; Corcoran et al. 2005; Stafford and Lattal 2009). Following the acquisition of contextual fear, markers like c-Fos show increased expression in the hippocampus (Huff et al. 2006) and hippocampal place cells show strong remapping to a context paired with shock in conditioning (Moita et al. 2004).

Our results showed that rapid reacquisition with and without prior extinction increased c-Fos expression in the dorsal hippocampus. Histone acetylation and c-Fos expression are involved in contextual fear and extinction (e.g., Stafford et al. 2012) and neurons within the dentate gyrus can be specifically associated with acquisition and extinction (Bernier et al. 2017). Additionally, many theorize that the hippocampus is a site important for indexing episodic memories and thus may be critical for relearning contextual fear in addition to initial learning (Teyler and Rudy 2007). Some studies have found that the hippocampus is necessary for rapid reacquisition for the memory of the spatial location of a food reward in a cross maze (Winocur et al. 2005) and molecular processes within the hippocampus are important for reacquisition of fear (Bevilaqua et al. 2005; Motanis and Maroun 2012).

The dentate gyrus was the site of most c-Fos-positive cells among the hippocampus subregions, suggesting the importance of the DG to contextual fear. Unlike the hippocampus as a whole, the DG had significantly more c-Fos expression following acquisition compared to both reacquisition groups, suggesting that the DG, in particular, was critical for contextual fear conditioning. The DG is critical for pattern separation (Treves and Rolls 1994) and is the best-known site of adult neurogenesis in rats (Altman and Das 1965). There has also been evidence that the DG is necessary for the retrieval of contextual fear (Lee and Kesner 2002, 2004). Further, the DG tags and recruits adult-generated neurons to update and strengthen remote spatial memories (Trousche et al. 2009). It is also possible that the c-Fos activation seen in the DG and hippocampus as a whole is due to spatial recognition and processing rather than specific to spatial learning. The hippocampus has shown the expression of IEG activity markers in tasks that are known to be hippocampal-independent (Guzowski 2002; Kubik et al. 2007), but spatial processing and spatial learning are

intrinsically related and difficult to dissociate here. The inclusion of a handling control group mitigated some of the c-Fos expression due to nonlearning-specific hippocampal processes, but the handling animals were not exposed to the conditioning context and could not account for activation of place cells specific to the context. Thus, there are multiple mechanisms through which the hippocampus could be activated by conditioning and reconditioning.

The results for the BNST show that it is a region that is critical for both the acquisition and rapid reacquisition of contextual fear. First, the IHC showed that initial acquisition led to increased c-Fos-positive cells in the adBNST. The BNST has been implicated in fear and anxiety-like responding (Davis et al. 2010; Sink et al. 2013) and contextual fear conditioning (Sullivan et al. 2004; Waddell et al. 2006; Zimmerman and Maren 2011). There are different accounts for the role of the BNST in fear conditioning, including the idea that it is important for sustained fear responses that occur in response to long duration and unpredictable cues, or that it is involved in encoding prediction errors based on the temporal pattern of shock presentation (Fendt et al. 2003; Walker et al. 2003; Davis et al. 2010; Goode and Maren 2017). During contextual fear conditioning, the shock is not discretely signaled to cause animals to show sustained defensive behavior during continuous exposure with the best predictor of shock, the context. Thus, enhanced markers of activity in the BNST following contextual fear acquisition were expected, as the BNST as a whole had previously shown increased markers of metabolic activity following the expression of contextual fear (González-Pardo et al. 2012). Additionally, the adBNST is particularly important for anxiogenic behavior and stress responses (Herman et al. 1994; Kim et al. 2013).

Finally, it is important to note several caveats to using histone acetylation and c-Fos expression as markers of neuronal activity during reconditioning. First, a limitation with c-Fos is that its expression habituates after repeated exposure to inducible events like stress (Melia et al. 1994). Habituation may occur through either habituation of cellular expression of c-fos when a cell is reactivated by the same stimulus or by habituation of the population of cells activated by repeated stimulus presentation, such that the population of cells activated the second time is smaller (Melia et al. 1994; Watanabe et al. 1994; Girotti et al. 2006). Decreased c-Fos has been seen throughout the fear circuit following preexposure to a context, tone, or shock in cued fear conditioning (Radulovic et al. 1998). Thus, habituation could potentially occur during repeated context or shock exposure used in our experiments. This pitfall of c-Fos could also explain the findings in adBNST, as rapid reacquisition involved repeated exposure to the context and shock and could have habituated c-Fos expression. Extinction by its nature involves repeated exposure to a stimulus, so any postextinction phenomenon that is investigated is potentially impacted by c-Fos habituation. Our findings of reduced c-Fos expression in reconditioning relative to initial conditioning, therefore, need to be considered in the context of c-Fos habituation.

However, c-Fos did increase in the hippocampus following reacquisition. This outcome suggests that even if c-Fos did habituate, a region particularly important for contextual fear learning still revealed an effect. It will be important in future studies to investigate immediate-early genes that show less habituation with repeated treatment like Δ FosB (Kelz et al. 1999).

A caveat with using H4K8 acetylation as a measure of learning is that histone acetylation can be caused by different processes that involve transcriptional activation. For instance, H4K8ac is up-regulated in inflammation (Ashbrook et al. 2015) and antibody responses (Li et al. 2013). This alternative could be represented in the H4K8ac results, where there was a very large magnitude of staining within the mPFC, amygdala, and dBNST. The massive H4K8ac staining of these regions could have created a ceiling effect

of expression that prevented the discernment of reliable group differences.

The research described here is significant for understanding how postextinction conditioning sessions can strengthen the original associative memory in a persistent manner. We show that, as with other unmasking procedures such as contextual renewal, rapid reconditioning is associative and appears to be unaffected by the amount of extinction (Bouton et al. 2011; Willcocks and McNally 2011). Our immunohistochemical results suggest that there are overlapping, but potentially unique brain regions that mediate initial conditioning and postextinction reconditioning. Finally, these sorts of results have implications at the clinical level, where PTSD symptoms often return with a mild reconditioning episode, even after successful treatment (Paunovic and Öst 2001; Pizzimenti and Lattal 2015). Rapid reconditioning, therefore, is a useful process to investigate at both basic science and translational levels.

Materials and Methods

Animals and housing

Experimentally naive Male Long-Evans rats (Charles River Laboratories, Wilmington, MA) were purchased at 275–300 g (~9–11 wk of age) and were housed two rats to an individually ventilated cage. Cagemates received the same experimental treatment on a given day (e.g., conditioning or extinction sessions), but they were sometimes in different counterbalanced context groups (in Experiments 2 and 3). Rats in all experiments were kept on a reverse 12 h light–dark schedule (dark started at 06:00 and light started at 18:00). Food and water were available ad libitum and all behavioral experiments occurred in the dark phase from 09:00 to 15:00. The colony space was located within a suite that included an anteroom and three procedure rooms for behavioral experiments.

All animals were allowed to acclimate to the vivarium at Oregon Health & Science University (OHSU) for 7 d before any handling of behavioral procedures. The vivarium maintained a constant temperature ($22^{\circ}\text{C} \pm 1^{\circ}\text{C}$) and humidity (70%) in all animal rooms. All experimental procedures were approved by the OHSU Institutional Animal Use and Care Committee and were conducted in accordance with National Institutes of Health (NIH) “Principles of Laboratory Animal Care” (NIH Publication No. 86-23, revised 1985). Behavioral procedure rooms were within the same suite adjacent to the colony room.

Apparatus

Context A (CTX A)

Fear conditioning occurred in conditioning chambers (exterior dimensions: 31.8 cm L $\times$ 25.4 cm W $\times$ 26.7 cm H) housed within sound-attenuating chambers (Med Associates). The operant chambers were fixed with a grid floor set to deliver a 1 sec, 0.75 mA scrambled shock and a house light that illuminated to signal the start of the session. Before and after each round of behavior, the grid floors and chamber walls were cleaned with 95% ethanol. Animals were loaded into chambers in red light conditions to maintain the dark circadian cycle. For Experiments 2 and 3, the walls of the chamber were also fitted with horizontal black and white stripes to provide additional visual cues.

Context B (CTX B)

In Experiments 2 and 3, the second set of conditioning chambers were used as Context B. Context B differed from Context A in height (31.8 cm L $\times$ 25.4 cm W $\times$ 34.3 cm H), visual cues (two clear Plexiglas walls; two metal walls), cleaning solution (0.5% bleach), and loading light conditions (full light). These chambers were also equipped with levers, cue lights, and food hoppers (all of which were inactive). The chambers were fixed with the same model grid floors as CTX A set to deliver a 1 sec, 0.75 mA scrambled

shock and a house light that illuminated to signal the start of the session.

Habituation

Prior to fear conditioning, all animals were habituated to transport to the anteroom and handling for 3 d.

Freezing assessment

The level of contextual fear conditioning was assessed by the amount of freezing, the natural conditioned response to a context associated with shock (Bolles and Collier 1976). Freezing was hand scored by visual time sampling of each animal every 8 sec. The scorer was blind to treatment assignments except in cases where the differences were obvious (e.g., conditioning vs. extinction; different session durations).

Contextual fear conditioning and extinction

Rats were run through a modified version of the rapid reacquisition procedure we have previously used in mice (Williams and Lattal 2019). For all experiments, initial conditioning was a 12-min session, in which rats received four unsignaled footshocks (1 sec, 0.75 mA) at 2.5, 5, 9, and 11.5 min into the session. Extinction consisted of a 24-min nonreinforced exposure to the fear-conditioning context in all experiments, except for Experiment 4 (see below). Reconditioning was a single conditioning session that consisted of 3 min session with a single unsignaled footshock (1 sec, 0.75 mA) delivered 2.5 min into the session. Postreconditioning tests were 24-min nonreinforced exposure to the fear-conditioning context. All sessions occurred at the same time of day separated by 24 h.

Experiment 1: reconditioning after 2 or 12 extinction sessions

Twenty-four rats were divided into three groups: reconditioning following 12 extinction sessions (Group REC 12 EXT), reconditioning following two extinction sessions (Group REC 2 EXT), and initial conditioning (COND). All treatments occurred in Context A.

REC 12 EXT received conditioning on Days 1 and 2 (Phase 1) followed by 12 days of extinction on Days 3 to 14 (Phase 2). REC 2 EXT received conditioning on Days 11 and 12 (Phase 1) and extinction on days 13 and 14 (Phase 2). This behavioral schedule allowed groups to receive different amounts of extinction (12 vs. 2 d), but have the final 2 extinction sessions, reconditioning, and testing on the same days.

Day 15 was a reconditioning session (Phase 3) followed by the postreconditioning tests on Days 16 and 19 (Phase 4). The behavioral schedule is summarized in Figure 1A. All tests were 24 min.

Experiment 2: effects of a postextinction US in a different context

Thirty-two rats were subdivided into four groups that received different treatments: reconditioning following 3 sessions of extinction (Group REC 3 EXT, $n=8$), reconditioning following 6 sessions of extinction (Group REC 6 EXT, $n=8$), reconditioning in a different context following moderate extinction (Group SWITCH 3 EXT, $n=8$), and conditioning with the single shock for the first time (Group COND, $n=8$). The context was counterbalanced in each group, with half of each group run in CTX A ($n=4$ /group) and CTX B ($n=4$ /group) as their original conditioning context. For the purposes of the simplified behavioral schedule in Figure 1A and the discussion of the methods and results, the original context is referred to as CTX A and switched context is CTX B.

Phase 1: conditioning

Days 1 and 2 (Conditioning) consisted of conditioning sessions (12 min, four footshocks) for Group REC 6 EXT in CTX A. All other groups were transported to the anteroom and handled. On Days

4 and 5, Groups REC 3 EXT and SWITCH 3 EXT received conditioning in CTX A.

Phase 2: extinction

Twenty-four minutes extinction sessions occurred on Days 3 to 8 (REC 6 EXT) or Days 6 to 8 (REC 3 EXT and SWITCH 3 EXT) in CTX A. This behavioral schedule allowed the extinction groups to receive different amount of extinction (6 vs. 3 d of extinction), followed by reconditioning and testing on the same days.

Phase 3: reconditioning

All groups received reconditioning on Day 9. For groups REC 3 EXT and REC 6 EXT, reconditioning occurred in CTX A. For SWITCH 3 EXT, reconditioning occurred in CTX B.

Phase 4: tests

Day 10 was a postreconditioning test in the original conditioning context (CTX A; Test 1) and Day 11 was a test in the switched context for all groups (CTX B; Test 2). On Day 12, another postreconditioning test occurred in the original context (CTX A; Test 3) to test for long-term retention. Tests 1 and 2 were 24 min and Test 3 was 12 min.

Experiment 3: reconditioning with matched US and context exposure

Thirty-two rats were subdivided into four groups ($n=8$ /group). Group REC SAME received 2 sessions of initial conditioning (four shocks in 12 min, as in Experiments 1 and 2) and 3 sessions of extinction (nonreinforced 24-min context exposures) in Context A; Group REC DIFF received conditioning and extinction in Context B; Group COND SAME received equivalent nonreinforced exposure to Context A (matched to acquisition durations of two 12-min exposures and extinction durations of three 24-min exposures); Group COND DIFF received an identical amount of nonreinforced exposure to Context B. Twenty-four hours after the final extinction/context exposure session, all groups received a single weak conditioning session in Context A (3 min exposure with a shock after 2.5 min, as in Experiments 1 and 2). All groups received a nonreinforced 24-min test session in Context A 24 h after the weak conditioning session. The identities of Contexts A and B were counterbalanced.

Experiment 4: reconditioning after short or long extinction sessions

Sixteen male naïve Long-Evans rats on a Long-Evans Th-Cre^{+/−} background were used in this experiment separated into two groups that differed in extinction session length: reconditioning following 24-min extinction sessions (24 MIN, similar to reconditioning groups above) and reconditioning following 6 min extinction sessions (6 MIN).

Days 1 and 2 consisted of conditioning (Phase 1). For Group 24 MIN, Days 3 to 6 consisted of extinction training as described above (24 min of exposure to the context without shock). For Group 6 MIN, Days 3 to 6 also consisted of extinction, but these sessions were only 6 min of nonreinforced context exposure.

Day 7 was the reconditioning session (Phase 3) followed by the postreconditioning test on Day 8 for all groups (Phase 4). The behavioral schedule is outlined in Figure 4A.

Experiment 5: immunohistochemical characterization of rapid reconditioning

Methods

Animals and housing. Twenty-four male Long-Evans rats were purchased at 275–300 g (~9–11 wk of age) and were housed two rats to an individually ventilated cage.

Behavior

Behavioral schedule. For all experiments, conditioning was a 12-min session, in which rodents received four unsignaled footshocks (1 sec, 0.75 mA) at 2.5, 5, 9, and 11.5 min into the session. Extinction consisted of a 24-min nonreinforced exposure to the fear-conditioning context. Reconditioning was a weak conditioning session consisting of 3 min session with a single unsignaled footshock (1 sec, 0.75 mA) delivered 2.5 min into the session. All sessions were conducted at the same time of day separated by 24 h.

There were four groups that differed in conditioning history: reconditioning after 3 sessions of extinction (REC-EXT, $n=6$), reconditioning after no extinction (REC-NO EXT, $n=6$), conditioning only (COND, $n=8$), and a handled, behavior-naïve group (HAND, $n=6$). The full behavioral schedule is detailed in Figure 5A.

Phase 1: conditioning. Days 1 and 2 consisted of a conditioning sessions for REC-EXT and REC-NO EXT. All other groups were handled and transported to equate treatments.

Phase 2: extinction. On Days 3 to 5, REC-EXT received extinction training, while REC-NO EXT was handled and returned to the home cage without extinction training.

Phase 3: reconditioning. All groups received reconditioning, as described above, on Day 6. For groups REC-EXT and REC-NO EXT, Day 6 was a reconditioning session, but for COND this day was its first conditioning and exposure to the context. HAND was handled and returned to the home cage as in all previous phases. Fifty to ninety minutes following the end of Phase 3 all animals were sacrificed and perfused for brain collection.

Brain removal and cryoprotection

Animals were sacrificed via isoflurane until all breathing had stopped and then thoracotomy was used as a secondary form of euthanasia. Rats were then perfused with 4% paraformaldehyde (PFA) and phosphate-buffered serum (PBS). Brains were removed and placed in 4% PFA in PBS for no more than 24 h. For cryoprotection, brains were then placed in a solution of 20% sucrose and 0.1% sodium azide (NaN_3) in PBS until the brain was fully saturated with sucrose (generally 24 h). The brains were then transferred to 30% sucrose and 0.1% NaN_3 in PBS for no more than 1 mo before sectioning. The brains were sectioned at 35 microns and then placed in well plates of PBS and 0.1% NaN_3 until further processing via immunohistochemistry.

Immunohistochemistry (IHC)

Materials. Rabbit anti c-Fos IgG (F7799, Sigma-Aldrich, Saint Louis, MO) and goat anti-rabbit biotinylated IgG antibodies (BA-1000, Vector Laboratories) were purchased for c-Fos detection. Vectastain ABC Kit (PK-400, Vector Laboratories) and Metal Enhanced Diaminobenzidine (DAB) Kit (PI-34064, Fischer Scientific) were used for immunoreaction detection.

Rabbit anti H4K8 Acetyl-Histone H4 Lys8 (2594S, Cell Signaling, Danvers, MA) and goat anti-rabbit IgG F(ab')₂-Alexa Fluor 488 (Thermo Fisher Scientific) antibodies were purchased for H4K8ac histone acetylation detection.

Tissue selection for processing. Due to a large amount of tissue collected to include all regions of interest, not all of the tissue could be processed at once and multiple rounds of IHC were run. To have a balanced representation, tissue was processed by region in which all animals were included (e.g., every other slice of mPFC for each animal was processed simultaneously).

DAB IHC schedule. Procedures followed those described by Weitemier and Ryabinin (2004). All incubations were done at room temperature on a plate rotating at ~40 rpm. Slices in 12 well plates and net wells were first treated with 3 × 5 min washes in PBS to wash away NaN_3 . A 15 min incubation in 0.3% hydrogen peroxide in PBS blocked endogenous peroxidase activity and was followed by three additional PBS washes. The slices were then incubated in a blocking solution for 4 h, which consisted of a 1:10 solution of normal goat serum (NGS, S-1000, Vector Laboratories) into 0.1% triton in PBS (PBS/Triton). NGS was the blocker of choice in these molecular assays due to the animal (goat) in which the secondary antibody was raised. Following blocking, the slices were in-

cubated overnight (~15 h) in primary antibody, anti c-Fos rabbit IgG, that was at a concentration of 1:15000 in PBS/Triton/bovine serum albumin (BSA).

The following day commenced with 3 × 5 min PBS washes and subsequently with the secondary antibody incubation. The secondary antibody solution consisted of 1:200 concentration of biotinylated goat anti-rabbit IgG in PBS/Triton. This incubation lasted for 1 h and was followed with 3 × 5 min PBS washes.

Next, a Vectastain ABC kit was used to bind to secondary-antibody-bound cells via a biotin/avidin interaction. The ABC solution was a 1:200 concentration of both chemicals A (avidin) and B (biotin; the exact composition of which is proprietary information) into PBS/triton. This mixture was made 30 min prior to application and the incubation lasted for 1 h. This step was followed by PBS washes and the application of a 1× solution of diaminobenzidine (DAB) in peroxidase (H_2O_2) buffer that provided intense coloration of immunopositive cells. Unlike previous steps, this incubation was not done on the rotating plate. The incubation time in DAB was around 1 min, a time chosen from pilot data for the optimal for strong signal and minimal background (data not shown). This incubation time was kept consistent for all plates. Immediately after DAB incubation, the wells were placed into the ultraviolet (UV) purified water to stop the reaction and prevent the tissue from saturating.

The slices were then placed onto glass Superfrost slides (Fisher Scientific) via a mounting solution (composed of UV purified water, gelatin, acetic acid, and 95% ethanol) and allowed to dry overnight. After drying, the slides were dehydrated in sequential steps of ethanol at increasing concentrations: 10 min in 70%, 95%, and then 100% ethanol. Next, the slides were dehydrated in the final step by submersion into Citrasolv (Fisher Scientific) and immediately coverslipped with glass covers and Cytoseal 60 mounting media (Fisher Scientific).

Fluorescent IHC schedule. Fluorescent IHC was similarly run as DAB IHC above with several exceptions. Well plate IHC was employed again and slices were first treated with 3 × 10 min washes in 0.1 M Tris washes to wash away NaN_3 . Slices were then incubated in 1% sodium borohydride in Tris for 30 min followed by Tris washes (at least three washes) until the sodium borohydride reaction was removed. Prior to the primary antibody, blocking was performed by incubating the slices in 5% NGS in a 0.3% Triton-Tris solution for 45 min. Then, slices received an overnight incubation in the primary antibody, 1:1000 rabbit anti H4K8ac, in Triton-Tris and 1% NGS.

The following day, the primary antibody was washed away in 3 × 10 min Tris washes. Another round of blocking (45 min in 5% NGS) occurred prior to secondary antibody incubation. The secondary antibody incubation was 1 h submersion in 1:1000 goat anti-rabbit Alexa Fluor 488 and 5% NGS in Triton-Tris. During and following the secondary antibody, slices were protected from light at all times. The slices were then washed in Tris and 3 × 5 min in PBS. Following washes, slices were mounted on glass Superfrost slides in the mounting solution used above. Slices were not dehydrated, but instead cover-slipped with Vectashield mounting medium with DAPI (H-1200, Vector Laboratories) shortly after mounting on the slide. DAPI labeled the neurons with a fluorescence of 360 nm.

Tissue imaging and cell counting. The processed tissue was imaged using the Leica DM4000B microscope (Leica Microsystems Inc.) on brightfield settings for DAB or with laser light set to excite Alexa Fluor 488 for fluorescent markers. Each image was taken at a consistent intensity, field diaphragm, aperture, and magnification for each image and region ($I=8$; $A=25$; $FD=32$ for DAB and $I=100$; $FD=6$; $\Sigma=100\times$ for fluorescence). All images used for cell counting were taken with a 10× objective, except for the hippocampus, which required a 5× objective to capture the region. Each image was collected using QImage Micro-Publisher 3.3 RTV camera and software (QImaging) at the same exposure (exposure=84.1 msec for DAB and exposure=1.87 for fluorescence).

Each image was analyzed using ImageJ software (NIH). The images were adjusted through a threshold procedure, in which images were converted to an 8-bit image prior to the application of a threshold mask. The threshold mask was chosen using neighboring brain regions with only nonspecific background staining as control regions. A consistent threshold range (for DAB, 80–120; for fluorescence, 20–50 on a dark background) and circularity (0.7–1.00) were used throughout the regions. The lower limit of pixels required for a stained cell depended on the region, but was consistent within each region (60 pixels for mPFC regions, 50 pixels for amygdala and BNST, and 15 pixels for the hippocampus because the images were at a lower magnification).

Both whole regions (except the amygdala) and subregions were analyzed for group differences. The chosen location (relative to bregma) for each subregion was: IL (3.00 mm); PrL (3.35 mm); adBNST—dorsal subregion of anterior BNST (0.00 mm); avBNST—ventral subregion of anterior BNST (0.00 mm); CeA (−2.76 mm); BLA (−2.76 mm); LA (−2.76 mm); CA1 (−3.00 mm); CA2 (−3.00 mm); CA3 (−3.00 mm); DG (−3.00 mm). The Paxinos–Watson rat atlas was used to confirm the bregma location of slices and only slices within 0.35 mm of the bregma location listed above were considered for assessment of staining. Two to four slices were counted and averaged for each animal to get the average amount of target protein expression captured by IHC. Due to the loss of integrity of the tissue of a few animals, the total numbers of animals per group for each region are shown in Tables 1 and 2. For H4K8ac analyses, the CeA tissue was too degraded to include in analyses.

Statistics

R-Studio and GraphPad Software Prism 6 were used to run all statistics and create figures, respectively. Extinction sessions were generally analyzed by mixed analysis of variance (ANOVA) in which Group was the between subjects measure, and Extinction Session and Session Time Block were the within-subjects factors. Reconditioning was analyzed with a two-way RMANOVA comparing the average percent freezing in the 30 sec pre- and postshock (Time) by Group. Test sessions were analyzed with a one-way ANOVA (or *t*-test, if applicable) with percent freezing in time blocks within the session as the dependent measure and Group as the main factor. c-Fos or H4K8ac detection was analyzed with a one-way ANOVA with Group as the main factor and average protein counts per animal as the dependent measure. Any failure to meet the homogeneity of variances criterion for an ANOVA or *t*-test (as measured by the Brown–Forsythe Levene's test) was accounted for using a Welch correction. If significance was found for main effects or interactions, Tukey's HSD tests (or Games–Howell for unequal variances among groups) were used for simple comparisons between groups and sessions. For all statistical tests, significance was set at $\alpha = 0.05$.

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