Nucleus reuniens is required for encoding and retrieving precise contextual fear memories in rats

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Running title: Nucleus reuniens and memory precision

Conflict of Interest: The authors declare no competing financial interests.

Acknowledgements: This work was supported by grants from the McKnight Endowment for Neuroscience (McKnight Memory and Cognitive Disorders Award to SM) and the National Institutes of Health (R01MH065961) to SM. The authors would like to thank Kelsey J. Clement for his technical assistance.

Author contributions: KR and SM designed the experiments, analyzed the data and wrote the manuscript. KR, JJ and RR collected the data.

Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon request.

Abstract

The nucleus reuniens (RE) is a ventral midline thalamic nucleus that interconnects the medial prefrontal cortex (mPFC) and hippocampus (HPC). Considerable data indicate that HPC-mPFC circuits are involved in contextual and spatial memory, however, it is not clear whether the RE mediates the acquisition or retrieval of these memories. To examine this question, we inactivated the RE with muscimol before either the acquisition or retrieval of Pavlovian fear conditioning in rats; freezing served as the index of fear. We found that RE inactivation before conditioning impaired contextual freezing during retrieval testing in the conditioning context. Interestingly, this impairment was absent when retrieval testing was also conducted after RE inactivation. Inactivation of the RE alone did not affect the expression of context freezing, though it did increase the generalization of freezing to a novel context. Muscimol inactivation of the RE did not affect either the acquisition or expression of auditory fear conditioning. Together, these data reveal that the RE supports the encoding of precise contextual memories that allow discrimination of dangerous from safe contexts. When the RE is inactive, however, alternate neural systems acquire an impoverished and state-dependent contextual memory that is only expressed when the RE is offline.

Key words: nucleus reuniens; thalamus; fear conditioning; retrieval; medial prefrontal cortex; hippocampus

Significance statement

The midline thalamic nucleus reuniens (RE) coordinates communication between the hippocampus and medial prefrontal cortex, brain areas critical for contextual and spatial memory. Here we show that temporary pharmacological inactivation of the nucleus reuniens impairs the acquisition and precision of contextual fear memories after Pavlovian fear conditioning in rats. Critically, however, inactivating the RE during retrieval restores contextual memory in rats conditioned after RE inactivation. These data reveal that the RE is required for encoding precise contextual memories to support the discrimination of safe and dangerous contexts.

Introduction

The nucleus reuniens (RE) is a ventral midline thalamic nucleus that interconnects the medial prefrontal cortex (mPFC) and hippocampus (HPC) (McKenna and Vertes, 2004; Vertes et al., 2006). Recent work has shown that lesions or inactivation of the RE reduces synchrony between the mPFC and HPC (Preston and Eichenbaum, 2013; Jin and Maren, 2015; Hallock et al., 2016; Cholvin et al., 2017). Behaviorally, inactivation of RE produces deficits in tasks that require coordinated activity between the HPC and mPFC (Hembrook and Mair, 2011; Hembrook et al., 2012; Cholvin et al., 2013; Jin and Maren, 2015; Layfield et al., 2015). For example, RE inactivation impairs performance in spatial working memory task that requires delayed alternation in a T-maze, and these memory deficits are accompanied by reductions in theta coherence between the mPFC and HPC (Layfield et al., 2015; Hallock et al., 2016).

Deficits in spatial working memory associated with RE inactivation may be due to deficits in contextual processing associated with impaired HPC-mPFC interaction (Cassel and Pereira de Vasconcelos, 2015). Consistent with this possibility, chronic inhibition of synaptic transmission in the RE made with a virally expressed tetanus toxin (TetTox) disrupts contextual processing during Pavlovian fear conditioning in mice (Xu and Südhof, 2013). Although RE inactivation did not affect the acquisition or expression of freezing behavior in the conditioning context (or freezing to an auditory cue), it did cause a robust increase in freezing to a novel test context; optogenetic manipulation of RE activity during fear conditioning also impaired contextual discrimination. This impairment in contextual discrimination was also obtained after TetTox inhibition of the mPFC or its projections to RE. In addition to its role in contextual encoding, recent work also suggests a role for the RE in the consolidation of contextual memories (Vetere et al., 2017; Troyner et al., 2018). Together, these results suggest that the RE

is involved in encoding precise context representations that normally limit the generalization of fear from the conditioning context to other, dissimilar contexts.

Once learned, however, the retrieval of precise contextual memories does not appear to require the RE (Xu and Südhof, 2013). Yet there is considerable data in both animals and humans that HPC-mPFC interactions are involved in the retrieval of spatial and contextual memories (Orsini et al., 2011; Preston and Eichenbaum, 2013; Schlichting and Preston, 2016; Wang et al., 2016; Marek et al., 2018). Indeed, recent work has found that reversible optogenetic manipulations of the hippocampus yield robust deficits in both the encoding and retrieval of contextual fear memories (Goshen et al., 2011; Bernier et al., 2017). Moreover, RE inactivation produces robust impairments in spatial working memory (Griffin, 2015; Layfield et al., 2015; Hallock et al., 2016). Insofar as the RE plays a role in supporting HPC-mPFC interactions involved in both encoding and retrieving conditional memories, we hypothesize that reversible inactivation of the RE should affect both the acquisition and expression of contextual fear.

To examine this question, we functionally inactivated RE using intra-cranial infusions of muscimol (MUS, a $GABA_A$ agonist) during either the acquisition or retrieval of Pavlovian fear conditioning in rats. Retention tests were conducted in both the conditioning context and an alternate context to assess the influence of RE inactivation on context discrimination and generalization; both contextual and auditory freezing were assessed. Importantly, we included control groups in our design to determine whether state-dependent generalization deficits account for performance impairments in MUS-treated rats. Consistent with our hypothesis, we found that the RE is involved in both the encoding of retrieval of context representations that support contextual discrimination. Interestingly, deficits in contextual freezing in animals that underwent fear conditioning after RE inactivation could be rescued by inactivating the RE during

retrieval testing. This supports the view that the RE is a component of a hippocampal memory system that prioritizes the encoding of a configural representation of context that ultimately overshadows its underlying elements.

Materials and Methods

Subjects

One hundred and twenty-eight experimentally naïve adult male Long-Evans rats (Blue-Spruce; 200–224 g; 50–57 days old) were obtained from a commercial supplier (Envigo, Indianapolis, IN). The rats were individually housed in cages within a temperature- and humidity-controlled vivarium and kept on a 14:10 h light:dark cycle (lights on at 0700 hours) with *ad libitum* access to food and water. All experiments took place during the light phase of the cycle. Rats were handled for one minute per day for 5 days to habituate them to the experimenter before any surgical procedures or behaviors were carried out. All experiments were conducted at Texas A&M University with approval from its Animal Care and Use Committee.

Surgical procedure

One week before the behavioral testing, rats were anesthetized with isoflurane (5% for induction, ~2% for maintenance), and placed into a stereotaxic instrument (Kopf Instruments). An incision was made in the scalp, the head was leveled, and bregma coordinates were identified. Small holes were drilled in the skull to affix three jeweler's screws and to target RE using a cannula (8 mm, 26 gauge; Plastics One) above the RE. A single guide cannula was implanted at a 10° angle

on the midline (A/P: -2.05 mm, M/L: -1.0~1.05 mm, D/V: -6.6-6.8 mm from dura; coordinates were measured from bregma). The cannula was affixed to the skull with dental cement, and a stainless-steel dummy cannula (30 gauge, 9 mm; Plastics One) was inserted into the guide cannula. Rats were allowed to recover for a period of 7 d after surgery before behavioral testing during which the dummy cannulae were replaced twice.

Drug infusions

For RE microinfusions, rats were transported to a prep room in the laboratory using white buckets (5-gallon) filled with a layer of bedding. Dummies were removed and stainless-steel injectors (33 gauge, 9 mm) connected to tubes was inserted into the guide cannulae for intracranial infusions. Polyethylene tubing connected the injectors to Hamilton syringes (10 μl), which were mounted in an infusion pump (Kd Scientific). Infusions were monitored by the movement of an air bubble that separated the drug or saline solutions from distilled water within the polyethylene tubing. All infusions were made approximately 10 min before both conditioning and retrieval testing sessions. Muscimol (MUS) was diluted in sterile saline (SAL) to a concentration of 0.1 μg/μl. Infusions were made at a rate of 0.1 μl/min for 3 min (0.3 μl total, 0.03 μg muscimol) and the injectors were left in place for 2-3 min for diffusion. After infusions, clean dummies were inserted into the guide cannula and the animals were transported to chambers for the behavioral sessions.

Behavioral apparatus and procedure

Sixteen identical rodent conditioning chambers (30 × 24 × 21 cm; Med-Associates, St Albans, VT) were used in all behavioral sessions. Each chamber consisted of two aluminum sidewalls, a Plexiglas ceiling and rear wall, and a hinged Plexiglas door. The floor consisted of 19 stainless

steel rods that were wired to a shock source and a solid-state grid scrambler (Med-Associates) for the delivery of footshocks. A speaker mounted on the outside of the grating in one aluminum wall was used to deliver auditory stimuli. Additionally, ventilation fans and house lights were installed in each chamber to allow for the manipulation of contexts. Each conditioning chamber rests on a load-cell platform that is used to record chamber displacement in response to each rat's motor activity and is acquired online via Threshold Activity software (Med-Associates). For each chamber, load-cell voltages are digitized at 5 Hz, yielding one observation every 200 ms. Freezing was quantified by computing the number of observations for each rat that had a value less than the freezing threshold (load-cell activity= 10). Freezing was only scored if the rat is immobile for at least 1 sec. Stimuli were adjusted within conditioning chambers to generate two distinct contexts in two distinct behavioral rooms. For context A, a 15-W house light was turned on, and the room light remained on. Ventilation fans (65 dB) were turned on, cabinet doors were left open, and the chambers were cleaned with 1% ammonium hydroxide. Rats were transported to context A in white plastic boxes. For context B, house lights were turned off and fluorescent red room light was turned on. The cabinet doors were closed and the chambers were cleaned with 1.5% acetic acid. Rats were transported to context B in black plastic boxes.

The experiment was run in two replications with half of the animals undergoing a contextual conditioning fear procedure (n = 64) and the other half undergoing an auditory fear conditioning procedure (n = 64). There were no statistical differences (all F's < 1.2; p > 0.3) across these replications in the effects of RE inactivation on the acquisition or expression of freezing during conditioning or the context retrieval tests, so the data from these sessions were collapsed. Conditional freezing to the tone CS was assessed in only the cohort that underwent auditory fear conditioning.

Approximately one week after surgery, the animals were randomly assigned to one of four groups: SAL-SAL, SAL-MUS, MUS-SAL and MUS-MUS. On day 1 rats received microinfusions of either MUS or SAL and were subjected to either contextual or auditory fear conditioning in context A. The conditioning session consisted of a 3-min baseline followed by 5 footshocks unconditioned stimuli (US; 1mA 2 sec) equally spaced with 70 second intervals (ITI). In half the animals, the US was preceded by a 10-sec auditory conditioned stimulus (CS; 2 kHz, 80 dB). Twenty-four hours later, rats again received microinfusions of MUS or SAL and were placed in either the conditioning context (A) or a novel context (B) for a 10-min stimulus-free retrieval test to assess conditioned freezing and its generalization, respectively. Each rat was tested in each context in a counterbalanced fashion across two days and the infusion group assignment was the same for each test. Rats that received auditory fear conditioning were tested identically, except that five CS-alone test trials (30-sec ISI) were delivered after the 10-min baseline (generalization test) period in context B.

Histological procedures

Rats were overdosed with sodium pentobarbital (Fatal Plus, 100 mg/kg) and perfused transcardially with 0.9% saline followed by 10% formalin. The brains were extracted from the skull and post-fixed in a 10% formalin solution for 24 h followed by a 30% sucrose-formalin solution where they remained for a minimum of 72h. After the brains were fixed, coronal sections (40 µm thickness) were made on a cryostat (−20 °C), mounted on subbed microscope slides, and stained with thionin (0.25%) to visualize cannula placements (see Figure 1A for sample cannula placement).

Data analysis

Three rats died or were sacrificed before completion of the experiment and were excluded from the analysis. Of the 125 remaining animals, fourteen were excluded due to poor cannula placements resulting in the following group sizes: SAL-SAL (n = 29), SAL-MUS (n = 27), MUS-SAL (n = 31), MUS-MUS (n = 24) for the conditioning session and context retrieval tests. Freezing to the tone was assessed in roughly half the number of subjects: SAL-SAL (n = 16), SAL-MUS (n = 13), MUS-SAL (n = 16), MUS-MUS (n = 11). All behavioral data (mean ± SEM) represent the average percentage of freezing behavior during one-minute intervals during each session. During the tone retrieval test, freezing was averaged across both the auditory CS and the subsequent 30-sec ISI (CS+ITI). Freezing during the CS+ITI period is highly correlated with freezing to the CS itself and is less susceptible to competition by active CS-elicited orienting responses. Two-way analysis of variance (ANOVA) and repeated-measures ANOVA were used to assess general main effects and interactions (α = 0.05). Post-hoc comparisons in the form of Fisher's protected least significant difference (PLSD) tests were performed after a significant overall F ratio for ANOVA.

Results

Inactivation of RE impairs acquisition of contextual conditioning and generalization of conditioned freezing

Figure 1 (A and B) illustrates the spread of fluorescently labeled muscimol (TMR-X) in the RE along with an illustration of cannula placements in all the animals included in the analyses (Figure 1C). During the fear conditioning session, rats receiving SAL or MUS infusions into the RE exhibited low levels of freezing behavior prior to the first conditioning trial and increased

their freezing behavior across the conditioning trials (Figure 2A); there were no differences between MUS- and SAL-treated in the levels of conditioned freezing. These observations were confirmed in a one-way repeated measures ANOVA that revealed a significant main effect of training trial [$F(5, 545) = 166.9$; $p < 0.0001$] with neither a main effect of drug [$F(1,109) = 3.56$; $p = 0.06$] nor a drug x trial interaction [$F(5,545) = 0.78$; $p = 0.57$]. Hence, RE inactivation did not affect the expression of post-shock freezing during the acquisition of Pavlovian fear conditioning.

Twenty-four and forty-eight hours later, all animals were again infused with either SAL or MUS and placed in either the conditioning context (A) or a novel context (B) for a 10-min retrieval test; the test order in the two contexts was counterbalanced and the infusion assignment for each test was the same. Importantly, this design allowed us to assess the state-dependent effects of RE inactivation on the acquisition, expression, and generalization of conditioned freezing. As shown in Figure 2B, rats conditioned after MUS infusions into the RE (MUS-SAL group) exhibited a substantial impairment in conditioned freezing in the conditioning context. Interestingly, this impairment was absent in animals both conditioned and tested after MUS infusions into the RE (MUS-MUS group). The relatively high level of freezing behavior in the MUS-MUS rats was not simply due to a nonspecific increase in freezing caused by RE inactivation insofar as SAL-MUS animals were no different from SAL-SAL or MUS-MUS animals. All of these observations were confirmed in a two-way repeated measures ANOVA that revealed significant main effects for conditioning drug [$F(1, 107) = 8.28$; $p = 0.004$], testing drug [$F(1, 107) = 6.04$; $p = 0.02$], and time [$F(1, 107) = 8.90$; $p < 0.0001$]. Moreover, there was a trend towards a significant interaction between conditioning and testing drug conditions [$F(1, 107) = 3.52$; $p = 0.06$] (Figure 2B). Post-hoc comparisons revealed that freezing among rats in

the MUS-SAL group was significantly lower than that in the SAL-SAL (p =0.0007), SAL-MUS (p=0.0002) and MUS-MUS (p=0.003) groups, which did not differ from one another. This reveals that RE inactivation impairs the acquisition of contextual fear conditioning and does so in a state-dependent manner; testing animals in the same state under which they were conditioned resulted in high levels of conditioned freezing.

In contrast, when the animals were tested for their generalization of fear to a novel context a different pattern of results emerged. As shown in Figure 2C, rats conditioned after MUS inactivation of the RE exhibited similar and low levels of freezing relative to animals conditioned under SAL. However, MUS infusions prior to the generalization test increased conditioned freezing. Interestingly, animals conditioned and tested under MUS (MUS-MUS) exhibited the highest level of freezing, and this was manifest early in the test session. Rats conditioned under SAL and tested under MUS (SAL-MUS) showed low levels of freezing at the beginning of the test that increased over the course of the test to approach those in the MUS-MUS group. These observations were confirmed in a two-way repeated measures ANOVA that revealed a significant main effect of test drug [$F(1,107) = 22.78$; $p < 0.0001$] and time [$F(9,963) = 7.65$; $p < 0.0001$]; there was no interaction between conditioning and test drug [$F(1,107) =1.1$; $p = 0.29$]. However, there was a significant interaction between test drug and time [$F(9,963) = 2.31$; $p = 0.014$], which indicates that MUS increased the generalization of fear (SAL-MUS), and served as a retrieval cue to support contextual freezing in rats conditioned after MUS infusions in the RE (MUS-MUS). Importantly, increases in freezing were not due to nonspecific effects of MUS on locomotion, insofar as MUS did not decrease locomotor (i.e., load-cell) activity during the pre-trial baseline period on the conditioning day, nor did it affect shock-elicited activity (data not shown).

RE inactivation impairs contextual discrimination

Because animals received the same drug infusions across both of the context tests, we were able to assess contextual discrimination using a within-subjects analysis. As shown in Figure 3A, rats in all the groups exhibited a contextual discrimination and exhibited lower levels of freezing in context B compared to context A. Consistent with this observation, a three-way repeated measures ANOVA with between-subject variables of conditioning and testing drug condition and a within-subject variable of test context revealed a significant main effect of test context [$F(1,107) = 80.48$; $p < 0.0001$]. Furthermore, there was a significant main effect of test drug [$F(1,107) = 17.42$; $p < 0.0001$], but not conditioning drug [$F(1,107) = 2.3$; $p = 0.13$], although there was a trend towards a significant interaction between the drug conditions [$F(1,107) = 3.48$; $p = 0.06$]. Close examination of these data reveals that the SAL-SAL animals exhibited the highest level of contextual discrimination relative to all the other groups. To examine this possibility more closely, we calculated a discrimination index by computing the ratio between the differences in the average freezing across the 10-min tests (context A - context B) and the total freezing in each context (context A + context B). As shown in Figure 3B, MUS infusions into RE resulted in lower discrimination scores whether the infusions occurred before conditioning or retrieval testing. A factorial ANOVA revealed a trend towards a significant main effect of conditioning drug [$F(1,107) = 3.62$; $p = 0.06$] and a significant main effect of drug during testing [$F(1,107) = 6.83$; $p = 0.01$]; there was no significant interaction between these factors [$F(1,107) = 0.16$; $p = 0.69$]. Hence, it appears that RE inactivation reduces contextual discrimination when infused either before conditioning or retrieval testing.

RE inactivation does not impair acquisition or expression of freezing to an auditory CS

Of the subset of animals that received auditory fear conditioning, we determined whether inactivation of RE causes deficits in acquisition or expression of fear to the tone CS. As shown in Figure 4A and B, conditional freezing to the tone was similar in all of the groups. Two-way repeated measures ANOVA revealed no significant main effects or interactions of drug on conditioning [$F(1,52) = 2.6$; $p = 0.12$] or testing [$F(1,52) = 1.77$; $p = 0.19$] across the entire test. Moreover, average freezing during the CS trials (Figure 4B) did not differ between the groups [conditioning, $F(1,52) = 3.47$; $p = 0.07$; testing, $F(1,52) = 1.05$; $p = 0.31$]. Overall, these results indicate that inactivation of RE does not cause deficits in either the acquisition or retrieval of fear to an auditory CS.

Discussion

The present study examined the role of the RE in the acquisition, expression, and generalization of conditioned freezing behavior in rats. We show that when RE is inactivated prior to fear conditioning, animals exhibit low levels of contextual freezing when later tested in the conditioning context. However, this acquisition deficit could be completely rescued by testing these animals under RE inactivation, indicating that the contextual memory acquired with the RE inactivated is acquired in a state-dependent manner. That is, the contextual memory acquired in animals conditioned after RE inactivation is not expressed when retrieval testing occurs with a functional RE; rather, this RE-independent memory is only expressed when the RE is again inactivated. Quite interestingly, RE inactivation alone prior to retrieval testing did not impair freezing in animals that received SAL infusions prior to conditioning. In addition, inactivation of the RE during retrieval testing increased fear generalization to a novel context and thereby

decreased contextual discrimination. Finally, RE inactivation during conditioning or retrieval testing did not cause deficits in freezing to an auditory CS.

Our results are consistent with previous findings that inactivation of the RE leads to deficits in acquisition of passive avoidance and consolidation of contextual fear memories (Davoodi et al., 2011; Vetere et al., 2017; Troyner et al., 2018). Furthermore, our results are consistent with work showing that inactivating synaptic inputs to the RE, particularly from the PFC, leads to context freezing that generalizes across contexts in mice (Xu and Südhof, 2013). That said, this report did not find that RE inactivation impaired the acquisition or expression of contextual freezing. However, the RE inactivation in this study was permanent (e.g., TetTox inhibition of synaptic neurotransmitter release). As we have shown here, memories encoded under RE inactivation can be retrieved as long as retrieval occurs when the RE is offline.

The fact that RE is involved in contextual fear conditioning is in line with previous work implicating the role of RE in HPC-dependent processes such as spatial and working memory (Layfield et al., 2015; Hallock et al., 2016). In these tasks, memory relies heavily on contextual processing. During contextual fear conditioning, for example, animals must encode a contextual representation that then comes into association with the aversive US. After acquisition, this memory supports conditioned freezing in the conditioning context and allows animals to discriminate the dangerous conditioning context from other, safe places. What might cause the contextual discrimination deficits observed after RE inactivation? One possibility is that animals without a functional RE use a non-hippocampal system to acquire an "elemental" representation of the context (Maren et al., 1997; Rudy and O'Reilly, 1999; Maren, 2001; Matus-Amat et al., 2004; Rudy and Matus-Amat, 2005). Bereft of a memory system that integrates multimodal sensory information into a unified, configural representation of context, animals with permanent

damage to the RE might associate only the most salient features of the conditioning experience (perhaps even the experimenter or transport cues) with shock. Elemental associations would readily generalize across test contexts and fail to support subtle discriminations between contexts. Consistent with this view, many investigators have found that permanent hippocampal lesions fail to affect contextual conditioning *per se* (Maren et al., 1997; Frankland et al., 1998; Cho et al., 1999), but impair contextual discrimination (Frankland et al., 1998; Cho et al., 1999).

It has previously been argued that the hippocampal memory system, within which the RE is a critical component (Cassel and Pereira de Vasconcelos, 2015; Griffin, 2015; Jin and Maren, 2015), functions to acquire configural representations of context that enable context conditioning and discrimination (Maren et al., 1997; Frankland et al., 1998; Rudy and O'Reilly, 1999; Matus-Amat et al., 2004). Interestingly, in the absence of the hippocampal system, animals can acquire context memories using a non-hippocampal system that associated context elements with an aversive US to yield conditional freezing. However, this impoverished representation of context leads to considerable generalization, particularly across highly similar contexts. Under normal circumstances, hippocampal-dependent configural leaning overshadows the elemental learning system, which results in the encoding of context memories that later require the hippocampal system for retrieval (Sutherland et al., 2010). In the context of the present results, this model suggests that the RE functions as a critical component of the hippocampal memory system involved in encoding precise contextual representations during fear conditioning. More specifically, our data reveal that 1) the RE functions to encode contextual memories that support contextual discriminations and 2) when conditioning occurs during RE inactivation, memories of conditioning are formed, but are only accessible when retrieved again under RE inactivation. Interesting, we also show that RE inactivation does not prevent the retrieval of contextual

memories *per se* and causes inappropriate and generalized fear to safe contexts. Altogether, these results are consistent with the proposal that conditioned freezing to an aversive context can be supported by either a configural representation encoded by the hippocampal system or by an elemental representation encoded outside the hippocampus (Maren et al., 1997; Rudy and O'Reilly, 1999; Anagnostaras et al., 2001; Chang and Liang, 2017). Configural representations normally overshadow the elemental representation of context to dominate performance during retrieval. Similar to hippocampal lesions, RE inactivation impairs configural encoding of context representations, rendering an elemental memory of the conditioning experience that is inhibited when the hippocampal system is active at the time of retrieval.

By this account, animals conditioned after RE inactivation encode an elemental representation of context (e.g., context *A'*) that comes into association with the US and is only retrieved when the animal encounters context *A'* in the future. After fear conditioning, contextual freezing in animals conditioned under RE inactivation will only be expressed in context *A'*, which requires RE inactivation to be experienced. Moreover, this account assumes that configural representations of the context, which are encoded by the intact brain, are not only insufficient to retrieve the elemental *A'* association, but in fact inhibit its retrieval. Ultimately, this leads to both deficits in the contextual freezing and poor contextual discrimination. Overall, the present data reveal that the RE is critical for contextual processes involved in fear conditioning and suggest that it is a critical hub for HPC-mPFC interactions involved in learning and memory.

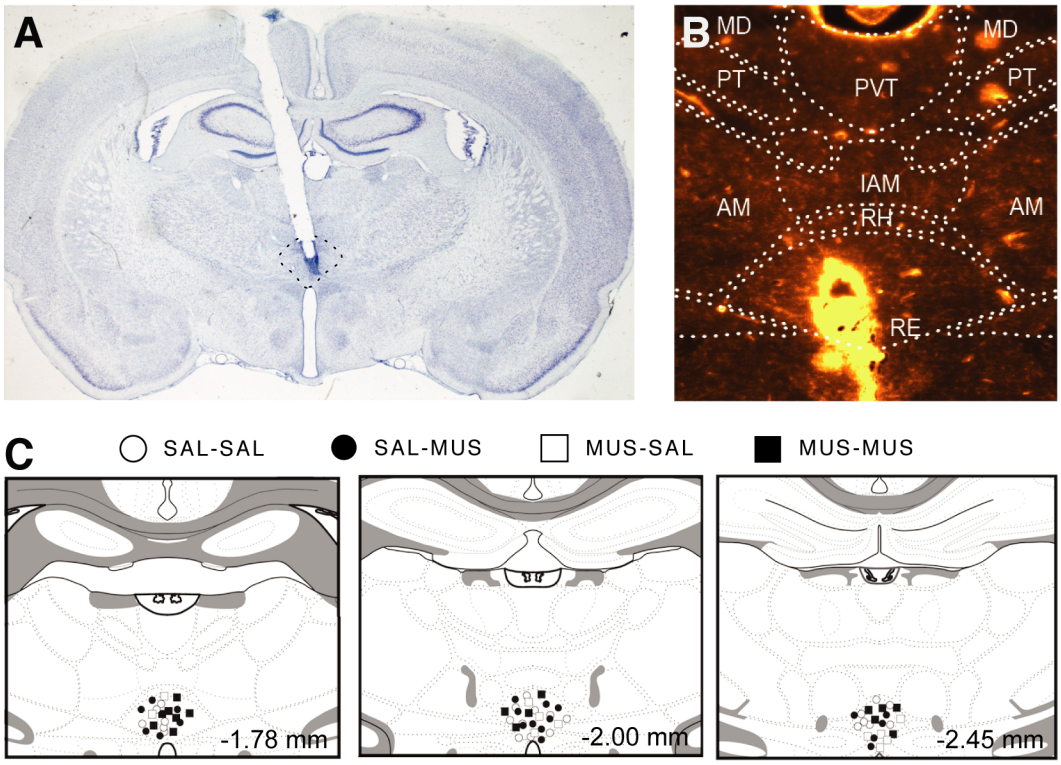

Figure 1: Cannula placements in nucleus reuniens (RE). *A*, Representative thionin-stained coronal section showing a midline cannula placement in the RE. *B*, Representative darkfield image showing diffusion of fluorescent muscimol (0.3 µl; TMR-X) in the RE. *C*, Cannula placements for all subjects that were included in the analysis across three different levels along the anterior-posterior axis. The distribution of cannula placements within the RE was similar across all of the groups.

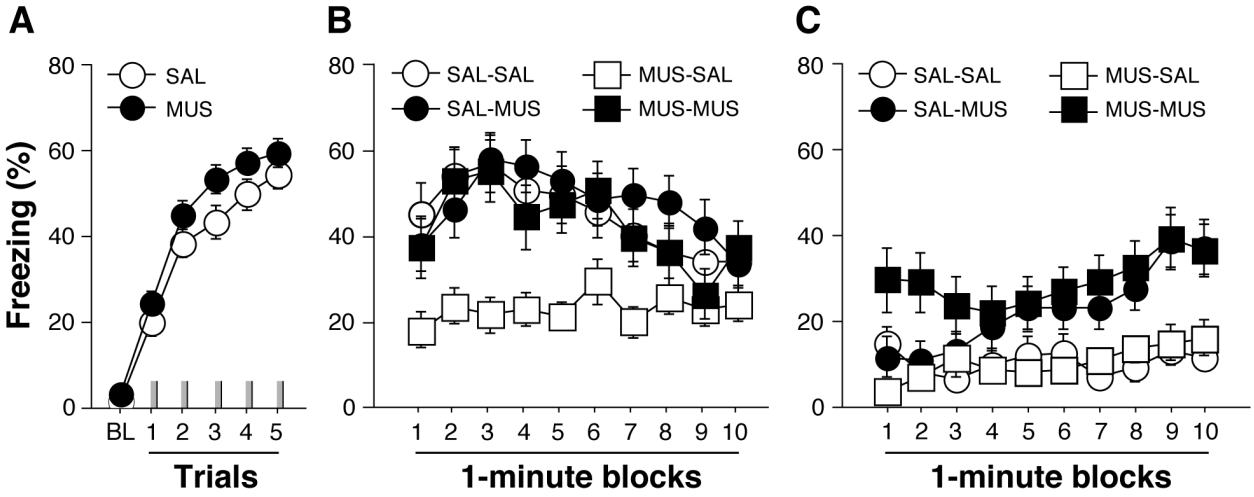

Figure 2: Muscimol inactivation of the RE results in a state-dependent impairment in contextual fear conditioning. *A,* Percentage of freezing during the 3-min baseline (BL) and 1-min ITI (intertrial interval) after each conditioning trial (indicated by gray hatch marks on the X-axis) in animals infused with either saline (SAL) or muscimol (MUS) in the RE. *B*, Percentage of freezing during the 10-minute retrieval test in the conditioning context. Animals conditioned after SAL or MUS infusions received the retrieval test after either SAL or MUS infusions in a factorial design that yielded four groups SAL-SAL, MUS-SAL, SAL-MUS, MUS-MUS. *C*, Percentage of freezing during the 10-minute retrieval test in a novel context (the order of the retrieval tests in *B* and *C* was counterbalanced); the groups are the same as those described in *B*. All data are shown as means ± SEMs.

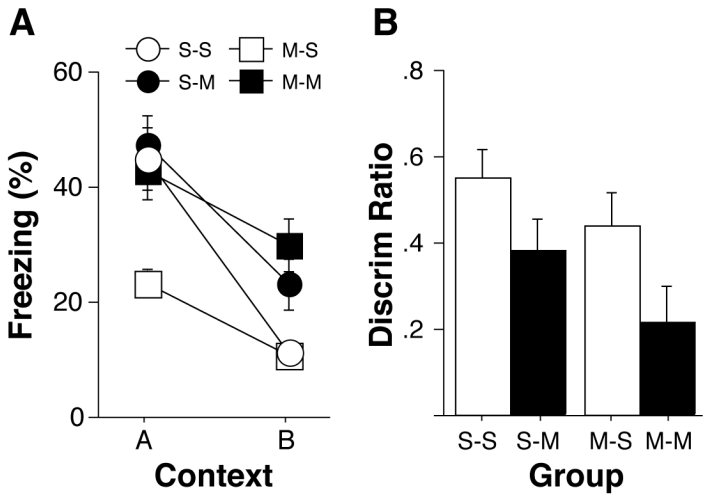

Figure 3: Muscimol inactivation of the RE impairs contextual discrimination. *A*, Average percentage of freezing across the 10-minute retrieval tests in the conditioning context (context A) and the novel context (context B) shown in Figure 2. The order of the two retrieval tests was counterbalanced and animals trained under saline (S) or muscimol (M) were tested in both contexts A and B under after either saline or muscimol infusions to yield the four groups described in Figure 2 (e.g., S-S, S-M, M-S, and M-M). *B*, Average discrimination ratios [(context A- context B)/(context A + context B)] calculated on freezing behavior across the 10-minute tests shown in *A*. All data are shown as means ± SEMs.

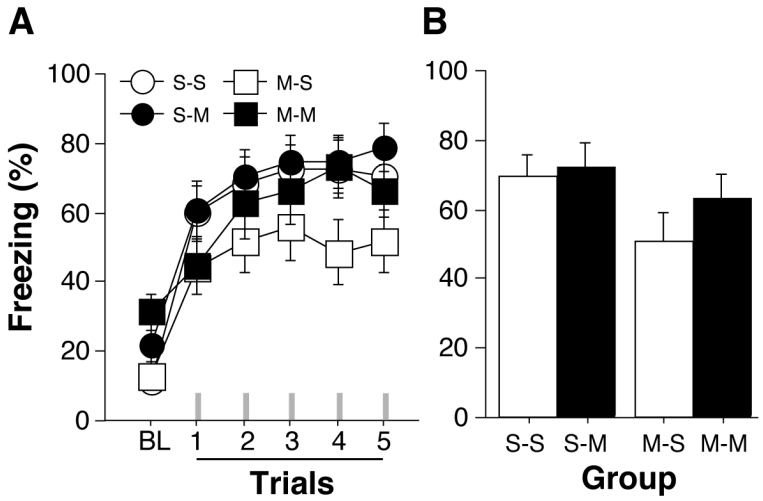

Figure 4: Muscimol inactivation of RE did not impair the acquisition or expression of freezing to an auditory conditioned stimulus (CS). *A*, Average percentage of freezing during a 10-min stimulus free baseline (BL) and after five tone presentations (indicated by the grey hatch marks on the x-axis) during an auditory retrieval test. Freezing during each trial represents the average freezing during the CS and intertrial interval. Animals received either saline (S) or muscimol (M) infusions prior to conditioning and retrieval testing in a factorial design to yield four groups (S-S, M-S, S-M, M-M). *B*, Average percentage of freezing across all five CS trials shown in *A*. All data are shown as means ± SEMs.

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