**REGULAR RESEARCH ARTICLE**

**Functional Reuniens and Rhomboid Nuclei Are Required for Proper Acquisition and Expression of Cued and Contextual Fear in Trace Fear Conditioning**

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**Abstract**

**Background:** The reuniens (Re) and rhomboid (Rh) nuclei (ReRh) of the midline thalamus interconnect the hippocampus and the medial prefrontal cortex. The hippocampus and medial prefrontal cortex are both involved in the acquisition of trace fear conditioning, in which a conditioned stimulus (tone) and an aversive unconditioned stimulus (footshock) are paired but separated in time with a trace interval. Earlier, we demonstrated that ReRh inactivation during trace conditioning impaired the acquisition of cued fear. In contrast, ReRh inactivation during both conditioning and test resulted in heightened fear to tones during retrieval. Because there was a generalized contextual fear on top of heightened fear to tones in the latter experiment, here we aimed to examine the specific importance of the functional ReRh in cued fear and contextual fear through introducing prolonged contextual exposure.

**Methods:** The ReRh were pharmacologically inactivated with muscimol (or saline as controls) before each experimental session.

**Results:** We showed that although ReRh inactivation before trace fear conditioning impaired the acquisition of cued fear, the animals still acquired a certain level of fear to the tones. However, without the functional ReRh throughout the entire behavioral sessions, these animals showed heightened contextual fear that did not decline much with the passage of time, which generalized to the other context, and fear to tones reoccurred when the tones were presented.

**Conclusions:** Our results suggested that functional ReRh are important for proper acquisition and expression of fear to context and tones acquired under trace procedure.

**Keywords:** Nucleus reuniens, rats, rhomboid nucleus, trace fear conditioning

**Introduction**

Trace fear conditioning is a procedure where animals are required to learn the association of an initially neutral conditioned stimulus (CS, e.g. a tone) and an aversive unconditioned stimulus (US, e.g., a footshock) with a trace interval in between (Pavlov, 1927; Connor and Gould, 2016). The presence of a temporal gap affects the mechanism of associative learning (Shors et al., 2000); more trials are needed to learn the association, and higher-order brain regions, such as the hippocampus (HPC), are recruited (Beylin et al., 2001; Shors et al., 2001; Czerniawski et al., 2009; Czerniawski et al., 2012). The ventral HPC projects robustly to the medial prefrontal cortex (mPFC); there is increasing evidence showing that the mPFC is involved in associative trace
fear memories (Gilmartin and Helmstetter, 2010; Gilmartin et al., 2013). Furthermore, it is indicated that the HPC-mPFC interaction is important for spatial working memory, episodic memory, and fear memory (Devito and Eichenbaum, 2011; Orsini et al., 2011; Preston and Eichenbaum, 2013; Marek et al., 2018). Together, the evidence suggests that the HPC, the mPFC, and the communication between the two are critical for emotion and memory processing.

The mPFC receives dense projection from the ventral HPC, but there is no direct projection back to the HPC (Vertes, 2004), suggesting that brain regions that interconnect the mPFC and HPC, such as the reuniens (Re) and rhomboid (Rh) nuclei (ReRh), perirhinal cortex (PRC), and entorhinal cortex, may have key roles in the coordination between the two (Naber et al., 1999; Delatour and Witter, 2002; Kajiwara et al., 2003; Agster and Burwell, 2013). The ReRh of the midline thalamus reciprocally project to both the mPFC and the dorsal and ventral HPC (Varela et al., 2014). Indeed, earlier studies revealed that inactivation or lesion of the ReRh disrupt mPFC-HPC synchronization during cognitive tasks (Hallock et al., 2016; Dolleman-van der Weel et al., 2019). The ReRh are important for the regulation of contextual fear memory (Sierra et al., 2017; Ramanathan et al., 2018). Inactivation of the ReRh led to anomalous generalization of fear to a novel context, which may be a result due to the disruption of the contextual processing relying on the circuit of the mPFC, HPC, and ReRh (Xu and Sudhof, 2013; Ramanathan et al., 2018). The ReRh are also critical for the learning of sequence memory with a temporal arrangement in it (Jayachandran et al., 2019). Because in trace fear conditioning there is a temporal component as well as the contextual cues presented in the background, we reasoned that the ReRh should have a role in this behavioral procedure.

Recently, we demonstrated that when the ReRh were inactivated during acquisition, early consolidation, or retrieval phase of trace conditioning, only the acquisition of cued fear was impaired such that the rats showed lowered cued fear during retrieval in a novel context (Lin et al., 2020). We also showed that when the ReRh were inactivated during both the acquisition and retrieval phase of trace conditioning, the rats showed heightened generalized fear and cued fear during the retrieval test in a new context (Lin et al., 2020). These data revealed that under normal circumstances, the ReRh are required for the encoding of cued fear in trace conditioning. However, 2 questions await to be further explored. Firstly, it is not clear whether the rats acquired any fear to the tones when the ReRh were inactivated during the acquisition. Secondly, it is also not clear how much of the fear the rats showed was attributed to the generalized fear and how much to the cued fear when the ReRh were off-line during both the acquisition and retrieval. To address these questions, we introduced “no conditioning” (NoCOND) controls in this study where these animals only received tones, but no footshocks, during conditioning (Experiment 1 and 2). If the animals acquired any fear to the conditioning context or tones under ReRh inactivation during trace conditioning, their fear level should lie between the NoCOND and conditioned controls during retrieval test. For animals without functional ReRh throughout the experimental sessions, we next examined whether their generalized fear in a novel context could decline if trained with weak conditioning and introduced with prolonged context exposure before tone presentations (Experiment 2). We aimed to decrease the contextual fear so that the level of fear to tones could be better evaluated. The ReRh were temporarily inactivated during only the acquisition phase or both the acquisition and retrieval phase of trace fear conditioning. Cued and contextual fear were assessed across behavioral sessions.

MATERIALS AND METHODS

Subjects

A total of 88 adult male Long-Evans rats (National Laboratory Animal Center, Taiwan) weighing 200–250 g (6–7 weeks old) at the beginning of the experiment were used. Animals were individually housed under a 12:12 hour-light/-dark cycle (lights on at 7:00 AM) with temperature (22 ± 1°C) and humidity (60–70%) controlled and had ad libitum access to food and water. Rats were handled for 10 s/d for at least 5 days before surgery. All procedures performed on the animals were conducted during the light phase of the cycle (9:00 AM to 5:00 PM) at National Yang Ming Chiao Tung University with approval from both the National Tsing Hua University and National Yang Ming Chiao Tung University Institutional Animal Care and Use Committees.

Surgery

Rats were anesthetized with i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and then placed into a stereotaxic instrument (Stoelting, IL, USA). Core body temperature was maintained at 37°C by a temperature-controlled heating pad (CWE, PA, USA). Small holes were drilled into the skull for cannula implant and 3 anchor screws. For drug infusion, 1 single stainless-steel guide cannula (26-gauge, 7.5 mm, Plastics One, VA, USA) was used to target the Re (relative to bregma: anterior-posterior –2.3 mm; medial-lateral +1.9 mm; dorsal-ventral –6.5 mm) at a 15° angle from the vertical midline. A representative image of Nissl-stained section for injection site is shown in Figure 1A. Afterward, the headstage was fixed with dental acrylic, and a dummy (33-gauge, extending 1.0 mm beyond the guide cannula, Plastics One, VA, USA) was screwed onto the guide. Carprofen (5 mg/kg) was s.c. injected before the animal was placed back in the home cage and was i.p. injected the following 2 days. Rats were allowed to recover for at least 5 days before any behavioral procedures, and the dummies were changed every day to prevent blockade of the cannula.

Significance Statement

Fear-related behavior is crucial for animal survival. However, inappropriate fear learning and expression may result in mental disorders in humans. In laboratory settings, trace fear conditioning is used to study the association of a tone and footshock with a temporal gap between. The literature revealed that the hippocampus and medial prefrontal cortex are required for the acquisition and expression of trace fear. The reuniens (Re) and rhomboid (Rh) nuclei (ReRh) serve as a hub between the two, but their role in this procedure is unclear. Here we reported that ReRh inactivation impaired the acquisition of trace fear conditioning. However, without functional ReRh, the rats showed heightened fear to the conditioning context that generalized to the other context as well as heightened fear to the tones. Our study revealed the critical role of the ReRh in acquisition and regulation of cued and contextual fear.
Drug Infusions

All rats were acclimated to the infusion procedures 1 day before the start of the behavioral experiments. Immediately before each behavioral procedure, intracranial infusions were performed. For drug infusion, dummies were first removed and injectors (33-gauge, extending 1.0 mm beyond the guide cannula, Plastics One, VA, USA) connected to Hamilton syringes through polyethylene tubes inserted into the guide cannulae. Infusions were conducted with a micro-infusion pump (Harvard Apparatus, MA, USA). GABAA receptor agonist “muscimol” (MUS, 0.1 mg/mL, Alfa Aesar, MA, USA) or vehicle (0.9% saline) was infused at the rate of 0.25 μL/min for 2 minutes (0.5 μL total), and the injectors were remained in place for another 30 seconds for drug diffusion. The dosage of MUS was chosen based on earlier studies for similar experimental purposes (Ramanathan et al., 2018). The effect of MUS lasts approximately 3 hours after the injection (van Duuren et al., 2007). Dummies were then inserted back into guide cannulae, and animals were then transported to the chambers for training and test.

Regular MUS was used in all the behavioral studies. Nonetheless, an example of the diffusion using fluorescent-labeled TMR-X MUS (Life Technologies, CA, USA) in the ReRh is shown in Figure 1B. Notably, the inactivation was limited to the injection center of the Re and the above Rh, whereas the more lateral portions of the Re were not affected.

Behavioral Apparatus

All behavioral sessions were conducted in 4 identical conditioning chambers (Med Associates, VT, USA) housed in sound-attenuating cabinets. Two distinct contexts were generated. For Context A, the room light and chamber lights remained on, the cabinet doors were half open, and the fans worked to produce background noise. The chambers were cleaned with acetic acid (1%), which was also poured into the pans beneath the rods to provide an odor. Animals were transported to the chambers in transparent cuboids. For Context B, acrylic plates and A-frames were inserted into the chambers. A faint red light was on to replace the room light, chamber lights and fans were off, and the cabinet doors were closed. The chambers were cleaned with ammonium solution (1%), which was also poured into the pans. Animals were transported to the chambers in buckets and covered with black sheets.

Behavioral Procedures

On each day, the order and group of the rats to go through the procedures were counterbalanced. Experiment 1—Rats were randomly assigned into 4 groups: NoCOND (with saline infusions), saline (SAL)-SAL, MUS-SAL, and MUS-MUS. Each group received micro-infusions of either SAL or MUS into the ReRh immediately before conditioning training and retrieval test in a 2-day procedure. On Day 1, auditory trace fear conditioning training was conducted in Context A. The conditioning session began with a 3-minute baseline (BL) followed by 10 trials of tone (CS; 20-second, 85 dB, 2 kHz)-footshock (US; 2-second, 1.0 mA) pairing with a 30-second trace interval between and a 240-second inter-trial interval (ITI). NoCOND rats received CSs only but no USs. On Day 2, a retrieval test was performed in Context B to minimize contextual fear. After a 3-minute baseline, all rats received five 20-second CS with an ITI of 60 seconds (Fig. 2A).

Experiment 2—Rats were randomly assigned into 1 of the following groups: NoCOND (with SAL infusions), SAL-SAL-SAL, MUS-SAL-SAL, and MUS-MUS-MUS. Each group received micro-infusions of either SAL or MUS immediately before the behavioral sessions in a 3-day procedure. The same settings for tone and footshock as in Experiment 1 were used. On Day 1, 5 trace conditioning trials with a 30-second trace interval and a 240-second ITI were given after a 3-minute BL in Context A. On Day 2, contextual fear was assessed by placing the animals back in Context A for 30 minutes. On Day 3, generalized contextual fear was assessed by placing the rats in Context B, followed by 30 twenty-second CS with an ITI of 60 seconds to assess cued fear (Figure 3A).

Histology

At the conclusion of the last behavioral procedure, all rats were killed by exposure to CO2 and then decapitated. Brains were removed from the skull and then fixed with 8% paraformaldehyde in 0.2 M phosphate buffer for at least 48 hours before being transferred into a 25% sucrose solution in 0.1 M phosphate buffer until saturated. Tissues were then sectioned coronally into 60 μm with a cryostat at –20°C and mounted onto subbed slides followed by Nissl staining to visualize the injection sites.

Statistics

All behavioral procedures were recorded using Video Freeze (Med Associates, VT, USA). Freezing level, which is widely used for the assessment of fear, has been used in previous studies [e.g., (Ramanathan et al., 2018)]. To analyze freezing level, the number of seconds freezing was recorded for each rat in each context. For each rat and context, the average freezing level across the last 3 minutes of each session was calculated. The overall freezing data were analyzed using one-way ANOVA followed by Bonferroni post-hoc tests if significant effects were found. The behavioral data were analyzed using one-way ANOVA followed by Bonferroni post-hoc tests if significant effects were found. The behavioral data were analyzed using one-way ANOVA followed by Bonferroni post-hoc tests if significant effects were found.
to assess the fear of animals (LeDoux, 2000), was presented as “immobility” because the NoCOND group never received the footshocks. “Immobility” was defined as consecutively observed movements below the motion threshold (program set at 100) for 1 second (video frame sampling at 0.2 seconds, e.g., at least continuous 5 frames below threshold) and was measured continuously during all of the behavioral sessions. The percentage of total observations in which immobility occurred at BL, to context (Experiment 2, in 5-minute blocks), and during CSs was calculated. These values were submitted to 2-way repeated-measures ANOVA with between-subject factor of “group” and within-subject factor of “trial” (as well as “5-minute block” and “5-minute block/5-trial block” in Experiment 2). If a significant F ratio in the ANOVA was obtained, Student–Newman–Keuls post hoc comparisons were performed. All data were calculated using SPSS (IBM, NY, USA) and presented as means ± SEs.

RESULTS

Experiment 1: ReRh Are Required for Normal Acquisition of Trace Fear Memory

Previously, we demonstrated that inactivation of the ReRh during conditioning impaired the acquisition of the trace fear, whereas inactivation of the ReRh immediately after conditioning or before test did not interfere with the early consolidation or
retrieval of cued fear. Moreover, inactivation of the ReRh during both conditioning and test resulted in heightened fear during retrieval in a novel context (Lin et al., 2020). In this experiment, the NoCOND control group was introduced in a 2-day procedure (Figure 2A) to address the question of whether the animals acquired any trace fear to tones when the ReRh were inactivated during acquisition phase.

**Histology and Final Groups**—A total of 40 animals were used in this experiment. We had difficulty to anesthetize 1 rat for surgery, and 1 rat died before the start of the behavioral procedure. Among the remaining animals, 5 rats were excluded due to cannula misplacements or inadvertent lesions. Two behavioral outliers (1 in the NoCOND group and 1 in SAL-SAL group, immobility level during test phase beyond 2 standard error compared with the group mean) were also excluded from further analyses. The final group sizes are NoCOND (n = 9), SAL-SAL (n = 8), MUS-SAL (n = 10), and MUS-MUS (n = 9). The placements of the injector tips for all the animals included in data analyses are summarized in Figure 2B.

**Behavior**—On Day 1 (Figure 2C, left), the animals received SAL (NoCOND, SAL-SAL) or MUS (MUS-SAL, MUS-MUS) microinfusions immediately before behavioral session on each day. In this experiment, the NoCOND control group was introduced in a 2-day procedure (Figure 2A) to address the question of whether the animals acquired any trace fear to tones when the ReRh were inactivated during acquisition phase.

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**Behavior**—On Day 1 (Figure 2C, left), the animals received SAL (NoCOND, SAL-SAL) or MUS (MUS-SAL, MUS-MUS) microinfusions immediately before the behavioral session. Other than the NoCOND group, the immobility levels increased rapidly from BL as the trials proceeded. There were significant main effects of “group” (F [3,27]=8.40, P<.001) and “trial” (F [10, 270]=32.61, P<.001) and a significant 2-way interaction between “group” and “trial” (F [30,270]=2.33, P<.001). Post hoc comparisons among groups revealed equivalent fear acquired for SAL-SAL, MUS-SAL,
and MUS-MUS groups (all Ps > .05), which were significantly higher than the NoCOND group (all Ps < .05).

On Day 2 (Figure 2C, right), the animals received SAL (NoCOND, SAL-SAL-SAL, MUS-SAL) or MUS (MUS-MUS) micro-infusions immediately before the retrieval test in a novel context. The NoCOND group remained a low immobility level throughout the test session, while the SAL-SAL group demonstrated fear response to the tones. Rats that underwent conditioning with ReRh inactivation showed a lower immobility than the SAL-SAL control group when tested with functional ReRh (the MUS-SAL group). Additionally, rats that underwent both the conditioning and retrieval test with ReRh inactivation (the MUS-MUS group) showed a relatively high BL immobility and an even higher level of fear to tones than SAL-SAL group. There were significant main effects of “group” (F [3,32] = 26.79, P < .001) and “trial” (F [5,135] = 11.30, P < .001) but no statistical difference in the 2-way interaction between “group” and “trial” (F [15,160] = 1.19, P = .29). Post hoc comparisons among groups revealed that fear level of the SAL-SAL group was significantly higher than the NoCOND group, while fear level of the MUS-SAL group was between the 2 and significantly differed from the SAL-SAL and NoCOND groups, respectively (all Ps < .05). Lastly, the MUS-MUS group had the highest immobility compared with all the other groups (all Ps < .05). Together, our results suggested that ReRh inactivation during the conditioning impaired, but did not totally abolish, the acquisition of trace fear conditioning; these rats still acquired certain level of cued fear. However, without functional ReRh throughout both behavioral sessions, there was a substantial up-shift in fear level during the retrieval test, consistent with our earlier study (Lin et al., 2020).

Experiment 2: Functional ReRh Are Required for Proper Expression of Contextual and Cued Fear

In Experiment 1, a context shift was introduced between conditioning (Day 1) and test (Day 2) with the hope to decrease the contextual fear. However, there was an up-shift in BL fear level of the MUS-MUS group, and this generalized contextual fear confounded with the interpretation of heightened cued fear to the tones. In this experiment, we aimed to examine whether contextual fear, generalized fear, and cued fear could decline with the passage of time. To achieve this goal, a 3-day procedure was performed (Figure 3A). The number of conditioning trials was decreased to 5 (weak training procedure) to weaken the strength of associative learning to both the tones and the conditioning context to prevent overtraining. Moreover, prolonged contextual exposures (30 minutes) were introduced aiming to decrease the fear to the conditioning context (Day 2) and the generalized contextual fear (Day 3) before tone test (Day 3).

Histology and Final Groups—A total of 48 animals were used in this experiment. One rat was killed before the start of the behavioral procedures because of the loss of the headstage. Among the remaining animals, 11 rats were excluded due to cannula misplacements or inadvertent lesions. The final group sizes were NoCOND (n = 9), SAL-SAL-SAL (n = 8), MUS-SAL-SAL (n = 10), and MUS-MUS-MUS (n = 9). The placement of the injector tips for all the animals included in data analyses are summarized in Figure 3B.

Behavior—On Day 1 (Figure 3C, left), the animals received SAL (NoCOND, SAL-SAL-SAL) or MUS (MUS-SAL-SAL, MUS-MUS) micro-infusions immediately before the conditioning. Other than the NoCOND group, the immobility levels dramatically increased from BL as the trials proceeded. There were significant main effects of “group” (F [3,32] = 49.79, P < .001) and “trial” (F [5,160] = 117.28, P < .001) and a significant 2-way interaction between group and trial (F [15,160] = 8.32, P < .001). Post hoc comparisons among groups revealed equivalent fear acquired for SAL-SAL-SAL, MUS-SAL-SAL, and MUS-MUS-MUS groups (all Ps > .05), which were significantly higher than the NoCOND group (all Ps < .05).

On Day 2 (Figure 3C, middle), the animals received SAL (NoCOND, SAL-SAL-SAL, MUS-SAL-SAL) or MUS (MUS-MUS-MUS) micro-infusions immediately before being placed back to the original conditioning context. The NoCOND group remained a low immobility level throughout the exposure session, while the SAL-SAL-SAL group demonstrated fear to the context. Rats that underwent conditioning with ReRh inactivation showed a lower immobility than the SAL-SAL-SAL control group when tested with functional ReRh (the MUS-SAL-SAL group). Additionally, rats that underwent both conditioning and context test with ReRh inactivation (the MUS-MUS-MUS group) showed a higher immobility than the SAL-SAL-SAL control group. These effects were robust during the initial 5-minute blocks. Toward the end of the exposure session, contextual fear slightly decreased in the SAL-SAL-SAL and MUS-MUS-MUS groups, but immobility slightly increased in the NoCOND group probably due to prolonged session time in the chambers that resulted in decreased exploration. There was a significant main effect of “group” (F [3,32] = 15.79, P < .001) and a significant 2-way interaction between “group” and “5-minute block” (F [15,160] = 4.40, P < .001). Post hoc comparisons revealed that both the SAL-SAL-SAL group and the MUS-SAL-SAL group and the MUS-MUS-MUS group had significantly higher immobility levels compared with the NoCOND group during the first three 5-minute blocks (all Ps < .05). However, the immobility levels of the MUS-SAL-SAL group were also significantly lower than the SAL-SAL-SAL group during the first two 5-minute blocks (both Ps < .05). Moreover, the MUS-MUS-MUS group had the highest immobility levels that were significantly higher than the NoCOND group during 5-minute blocks #1–4 and 6, the MUS-SAL-SAL group during the first three 5-minute blocks, and the SAL-SAL-SAL group during the first two 5-minute blocks (all Ps < .05). Together, these results revealed that ReRh inactivation during trace fear conditioning not only impaired fear acquisition to the tones (Experiment 1) but also impaired the fear acquisition to the conditioning context (this experiment). Moreover, without functional ReRh throughout both the conditioning and exposure sessions, there was also a substantial up-shift in fear level during context test, similar to the result of Experiment 1 on Day 2.

On Day 3 (Figure 3C, right), the animals received SAL (NoCOND, SAL-SAL-SAL, MUS-SAL-SAL) or MUS (MUS-MUS-MUS) micro-infusions immediately before the retrieval test. All animals were first placed into a novel context for 30 minutes before the tone presentations. The NoCOND, SAL-SAL-SAL, and MUS-SAL-SAL groups demonstrated low immobility, which increased in amplitude toward later 5-minute blocks, likely due to their familiarity to the chambers and therefore decreased exploration. In contrast, the MUS-MUS-MUS group showed a high level of generalized contextual fear initially, which did not decline with the passage of time. Moreover, cued fear to tones only reoccurred in the MUS-MUS-MUS group. There was a marginal main effect of “group” (F [3,32] = 2.75, P = .06), a significant main effect of “5-minute block/5-trial block” (F [11,352] = 29.74, P < .001), and a significant 2-way interaction between “group” and “5-minute block/5-trial block” (F [33,352] = 2.14, P < .001). Post hoc comparisons revealed that there were no statistical differences in immobility levels among the NoCOND, SAL-SAL-SAL, and MUS-SAL-SAL groups during any of the 5-minute blocks.
or 5-trial blocks (all Ps > .05). However, immobility levels of the MUS-MUS-MUS group were significantly higher than the other 3 groups during the first three 5-minute blocks and the first 5-trial block (all Ps < .05). Together, prolonged contextual exposure in the shifted context did not lead to the decrease of the generalized fear in the MUS-MUS-MUS group; this group demonstrated strong contextual generalization and was the only group that showed cued fear to tones at the first 5-trial block.

**DISCUSSION**

Using a pharmacological approach, the present study showed that pre-conditioning inactivation of the ReRh impaired the acquisition of trace fear. However, when the ReRh were also inactivated during retrieval, these animals showed an up-shift in contextual fear when placed back into the conditioning context. Moreover, these animals showed generalized contextual fear, as well as heightened cued fear, when placed into a novel context. Taken together, our results suggest that the ReRh are crucial for proper control of fear learning and expression under trace procedure.

The ReRh are necessary for a variable of memory and executive functions that rely on the HPC and the mPFC (Vienna et al., 2018; Jayachandran et al., 2019). It is worth noticing that the HPC and the mPFC are not required for the acquisition of delay conditioning (Phillips and LeDoux, 1992; Corcoran and Quirk, 2007) but are recruited in trace fear memory (McClelland et al., 1998; Gilmartin and Helmstetter, 2010). In our previous study (Lin et al., 2020), we have demonstrated that the ReRh were involved only in the acquisition, but not the retrieval, of trace fear memory. Based on this result, the focus of this study was to specifically inactivate the ReRh during the acquisition phase and then assess the retrieval of the contextual and cued fear with or without the functional ReRh using the pharmacological approach (MUS-SAL and MUS-MUS group in Experiment 1; MUS-SAL-SAL and MUS-MUS-MUS group in Experiment 2). Two control groups (NoCOND and SAL-SAL/SAL-SAL-SAL) were included to illustrate the fear learning and expression of normal animals, but the functional role of ReRh inactivation specifically during the retrieval phase was not examined again.

Freezing is commonly used as a measurement of fear level after conditioning (LeDoux, 2000). However, lack of movement other than breathing sometimes occurred, and therefore the inclusion of the NoCOND controls provided an assessment of random immobility for animals that never received tone-footshock pairings. Indeed, the immobility level of the NoCOND group was lower compared with other groups in general. Nonetheless, a trend of increase in immobility toward latter trials or minute-blocks was apparent in both experiments, especially when the session time was long. The animals may have become habituated to the chambers after the prolonged session time, and the active exploration may have decreased. Other than that, all of our experiments were conducted in the light phase of the light/dark cycle, and the nocturnal rats had relatively low locomotor activity during light phase (Klejbor et al., 2013). These factors may have led to the observed increase in immobility in the NoCOND group toward the end of the session. The inclusion of the NoCOND controls is important in that the immobility levels higher than NoCOND in all other groups were more reliably a result of associative learning and represented how much fear they acquired after trace conditioning to the context or the tones.

With the inclusion of the NoCOND controls, we can address whether the animals acquired any cued fear under ReRh inactivation during the acquisition phase. In Experiment 1, the MUS-SAL rats still acquired a certain level of fear to the tones, suggesting that the acquisition of trace fear was impaired, but not totally abolished. Because the ReRh inactivation was limited to the center of the local infusion site (Fig. 1B), the more lateral portions of the Re were not affected and might have supported the partial learning. Alternatively, although the ReRh are recruited during the acquisition of trace fear memory, other relay pathways may also contribute to this process. For example, the PRC also interconnects the HPC and the mPFC, and lesions of the PRC are shown to cause deficits in trace fear conditioning (Romanski and LeDoux, 1992; Phillips and LeDoux, 1995; Kholodar-Smith et al., 2008). It is possible that without the ReRh, other pathways may have compensated the initial acquisition of trace fear.

Previously, we have shown that when the ReRh were inactivated during both the acquisition and retrieval phases, the rats showed heightened generalized fear and cued fear during a retrieval test in a novel context (Lin et al., 2020). The generalized contextual fear before tone presentations left it unclear how much of the fear the rats showed was attributed to generalized fear and how much to cued fear. Similar results were replicated in Experiment 1 in that the BL immobility of the MUS-MUS group was high, and cued fear almost hit the ceiling level. Therefore, in Experiment 2, a weak training procedure and prolonged exposure to conditioning and novel contexts were introduced, aimed to decrease the generalized fear before the presentation of the tones. On Day 2, the SAL-SAL-SAL group showed higher immobility compared with NoCOND controls, suggesting that they acquired fear to the conditioning context. On Day 3, there was no difference in immobility level between the SAL-SAL-SAL and NoCOND groups during exposure to novel context, suggesting no generalized contextual fear. Unexpectedly, the SAL-SAL-SAL animals did not show any cued fear after tone presentations. One possibility is that in the trace procedure, the static contextual stimuli and discrete cues compete for associative strength with the US (Phillips and LeDoux, 1994), and the context served as a more reliable predictor because of the temporal gap in between the CS and the US, especially under the circumstance that these animals received only 5 tone-footshock pairings (weak training procedure). This notion was supported in our data that on Day 2, the SAL-SAL-SAL group demonstrated their fear to the conditioning context. Another possibility is that the prolonged exposure to the novel context made the context a safety signal, and the rats may have recognized that the context was different from the conditioning one and suppressed the fear expression when the CSs were presented. As for the MUS-SAL-SAL group, the rats still acquired a certain level of contextual fear (Day 2), although the learning was impaired compared with the SAL-SAL-SAL controls, consistent with the earlier report (Ramanathan et al., 2018). Moreover, because the acquisition of trace fear was impaired under ReRh inactivation during conditioning and no cued fear was acquired in the SAL-SAL-SAL group, we also did not detect any cued fear acquired in the MUS-SAL-SAL group (Day 3).

In Experiment 2, the animals with ReRh inactivation throughout the behavioral sessions (MUS-MUS-MUS group) expressed an abnormal regulation of fear. The introduction of weak training procedure and prolonged contextual exposures did not decrease the generalized fear as we aimed for. These animals showed inappropriately high contextual fear (Day 2) that generalized to a safe context (Day 3), consistent with our previous works (Lin et al., 2020) and others that investigated the role of the ReRh in context specificity of fear.
memory (Xu and Sudhof, 2013; Ramanathan et al., 2018). Our data, together with earlier reports, supported the notion that contextual fear learned without the hippocampal memory system is not well controlled (Goshen et al., 2011; Bernier et al., 2017). Moreover, fear to tones was observed only in the MUS-MUS-MUS group, suggesting that without the ReRh, cued fear became easier to provoke. Contextual and cued fear acquired under ReRh inactivation only reoccurred under ReRh inactivation is consistent with the idea of state-dependent learning and retrieval (Ramanathan et al., 2018; Lin et al., 2020). Other than that, the high immobility they expressed is not likely a result of locomotor deficit, but learning experience related, because ReRh inactivation did not increase immobility level during baseline before conditioning on Day 1 in both experiments. It is worth noticing that the fear level these animals expressed was higher than the SAL-SAL-SAL controls, suggesting that the ReRh are not passively passing on the information between the mPFC and the HPC. The mPFC and the HPC have opposite roles in generalized fear expression. Inactivation of the mPFC led to overgeneralization of fear, whereas inhibition of the ventral HPC neuronal activity reduced contextual fear generalization (Xu et al., 2012; Bian et al., 2019). The opposite roles of the mPFC and the HPC suggest that the indirect communications between the two is important, and the ReRh may have contributed to proper fear expression.

A limitation of this study is that only male rats were used. We noted that this design restricted our data representativeness to the entire population, especially when we tried to address issues related to anxiety-related disorders. Indeed, hormonal cycles in males and females may differentially impact behavioral regulations of learning and expression, and therefore future works including both male and female rats are necessary and critical to assess the gender differences.

Dysfunction of the HPC-mPFC circuit leads to cognitive impairments in many mental disorders, including schizophrenia and posttraumatic stress disorder (PTSD) (Small et al., 2011; Godsil et al., 2013). One central symptom of PTSD and many other anxiety-related disorders is the overgeneralization of fear (Dunsmoor et al., 2017). Patients with PTSD struggle to suppress cue-triggered fear in an otherwise safe environment (Mahan and Ressler, 2012). Altered function of the thalamus has been found to be related to PTSD (Yin et al., 2011). Using the trace fear conditioning paradigm, fear control that requires higher-order of brain circuit can be further explored. Studies into the ReRh of the ventral midline thalamus provide us with insights into the etiology of these disorders.

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Interest Statement

Dr Chang and Ms Wu reported no biomedical financial interests or direct conflicts of interest.

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