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Chemogenetic inactivation of the nucleus reuniens impairs object placement memory in female mice

Miranda R. Schwabe, Carnita M. Lincoln, Margaret M. Ivers, Karyn M. Frick *

Department of Psychology, University of Wisconsin-Milwaukee, Milwaukee, WI 53211, United States

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ABSTRACT

Episodic memory is a complex process requiring input from several regions of the brain. Emerging evidence suggests that coordinated activity between the dorsal hippocampus (DH) and medial prefrontal cortex (mPFC) is required for episodic memory consolidation. However, the mechanisms through which the DH and mPFC interact to promote memory consolidation remain poorly understood. A growing body of research suggests that the nucleus reuniens of the thalamus (RE) is one of several structures that facilitate communication between the DH and mPFC during memory and may do so through bidirectional excitatory projections to both regions. Furthermore, recent work from other labs indicates that the RE is necessary for spatial working memory. However, it is not clear to what extent the RE is necessary for memory of object locations. The goal of this study was to determine whether activity in the RE is necessary for spatial memory as measured by the object placement (OP) task in female mice. A kappa-opioid receptor DREADD (KORD) virus was used to inactivate excitatory neurons in the RE pre- or post-training to establish a role for the RE in spatial memory acquisition and consolidation, respectively. RE inactivation prior to, or immediately after, object training blocked OP memory formation relative to chance and to control mice. Moreover, expression of the immediate early gene EGR-1 was reduced in the RE 1 hour after an object training trial, supporting the conclusion that reduced neuronal activity in the RE impairs the formation of object location memories. In summary, the findings of this study support a key role for the RE in spatial memory acquisition and consolidation.

1. Introduction

Although the dorsal hippocampus has long been identified as a central area for the consolidation of spatial memories, numerous cortical and subcortical areas also play critical roles in this process. One key partner in the circuit mediating spatial episodic memories is the medial prefrontal cortex (mPFC), specifically the infralimbic (IL) and prelimbic (PL) subregions. In male rodents, coordinated activity between the DH and mPFC is necessary for many forms of episodic memory, including spatial, object recognition, temporal order, and contextual fear memory (Chao, Nikolaus, Lira Brandão, Huston, & de Souza Silva, 2017; Jin & Maren, 2015; Jones & Wilson, 2005; Kitamura et al., 2017; Maharjan, Dai, Glantz, & Jadhav, 2018; Warburton & Brown, 2015). Our laboratory has found that chemogenetic inactivation of the mPFC or DH in ovariectomized female mice impaired object recognition and spatial memory consolidation in the object recognition and object placement tasks (Tuscher, Taxier, Fortress, & Frick, 2018). Furthermore, simultaneous administration of chemogenetic activators that did not impair memory on their own blocked memory consolidation in these tasks, suggesting that concurrent activity in the DH and mPFC is necessary for object memory consolidation (Tuscher et al., 2018).

However, despite the demonstrated importance of DH-mPFC interactions for memory consolidation, the nature of the connections between the DH and mPFC is not fully understood. There are relatively few direct projections from the CA1 and subiculum regions of the DH to layers 1, 5, and 6 of the PL and IL regions of the mPFC (Hoover & Vertes, 2007; Jay, Thierry, Wiklund, & Glowinski, 1992; Verwer, Meijer, Uum, & Witter, 1997), although they have been shown to play a role in consolidation of fear memory (Ye, Kapeller-Libermann, Travaglia, Inda, & Alberini, 2017). Moreover, although there are sparse projections from the anterior cingulate region of the mPFC to CA1-CA3 that play a role in memory retrieval (Rajasethupathy et al., 2015), no direct projections have been identified from IL or PL to the DH. Due to the limited nature of direct connections between the DH and mPFC, numerous investigators have recently posited that the majority of communication from the mPFC to DH related to episodic memory occurs indirectly through other

* Corresponding author at: Department of Psychology, University of Wisconsin-Milwaukee, 2441 E. Hartford Ave., Milwaukee, WI 53211, United States.
E-mail address: frickk@uwm.edu (K.M. Frick).

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regions such as the entorhinal cortex, perirhinal cortex, and nucleus reuniens of the thalamus (RE) (Chao, de Souza Silva, Yang, & Huston, 2020; Dolleman-van der Weel et al., 2019; Jin & Maren, 2015).

In support of this hypothesis, recent work suggests that the RE is necessary for cognitive functions involving coordination between the mPFC and dorsal and/or ventral hippocampus, including spatial memory. In male mice, the RE was identified as critical among a network of brain regions that facilitate contextual fear memory, as inactivation with an hM4Di DREADD (Designer Receptors Exclusively Activated by Designer Drugs) immediately after contextual fear training impaired freezing during testing (Vetere et al., 2017). RE inactivation with muscimol before contextual fear training also impaired acquisition of fear memory in male rats (Ramanathan, Ressler, Jin, & Maren, 2018), supporting a key role for the RE in hippocampus-dependent learning. Consistent with this role, synaptic strength was increased in male mice at RE-mPFC and RE-CA1 synapses following trace eyeblink conditioning, another model of hippocampus-dependent associative learning (Eleore, López-Ramos, Guerra-Narbona, Delgado-García, & Izquierdo, 2011). This study also indicated involvement of the RE in object learning, as disrupting RE activity with high-frequency stimulation prior to training impaired object recognition memory in male mice (Eleore et al., 2011). Consistent with these findings, other data from male rats and mice show that permanent inactivation of the RE impairs multiple forms of spatial memory, including object placement in an open field (Jung, Huh, & Cho, 2019) and spatial working memory in a T-maze (Layfield, Patel, Hallock, & Griffin, 2015; Maisson, Gemzik, & Griffin, 2018).

Although these studies strongly suggest a role for the RE in hippocampal memory, none included females. This omission is notable because sex differences in the hippocampus and prefrontal cortex, and in various aspects of spatial memory, have been reported for many years (for reviews, see Hamson, Roes, & Galea, 2016; Jonasson, 2005; Koss & Frick, 2017). As such, it is essential to independently assess the brain circuitry underlying the neurobiology of learning and memory in both sexes, as sex-specific differences in the function or role of the DH, mPFC, or RE in memory may exist. Therefore, the goal of this study was to determine the extent to which the RE is essential for spatial memory, as assessed by the object placement task, in female mice. Although estrogenic effects on memory were not studied here, the female mice used were ovariectomized for consistency with our laboratory's previous episodic memory circuitry studies (Tuscher et al., 2018, 2019) and to set the stage for future studies investigating the role of the RE in estrogenic regulation of spatial memory in females. Here, we used the inhibitory κ-opioid DREADD (KORD) to inactivate the RE either prior to or immediately after object training, thereby allowing examination of a role for the RE in acquisition and consolidation of object location memories. Confirmation of this inactivation was assessed via immunohistochemical quantification of the immediate early gene EGR-1 in the RE. Based on the previous research in males described above and our data from ovariectomized female mice showing that pre- or post-training chemogenetic inactivation of the DH or mPFC impairs object placement memory (Tuscher et al., 2018), we hypothesized that RE inactivation would impair both acquisition and consolidation of OP memory. Consistent with this hypothesis, both pre- and post-training RE inactivation impaired OP memory in females, and subsequent RE inactivation reduced DH EGR-1 expression. Thus, these data support the involvement of the RE in spatial memory formation in female mice.

2. Materials and methods

2.1. Subjects

Female C57BL/6 mice (n = 48, Taconic, Cambridge City, IN) were 8–10 weeks old upon arrival in the lab. Mice were housed in groups of up to 5 per cage until the day before surgery, when they were separated and singly housed for the remainder of the experiment. Mice were maintained on a 12-hr dark/light cycle (lights on at 7 am) and given *ad libitum* access to food and water. All experimental protocols and procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2. Surgical procedures

A minimum of 4 days after arrival, mice were bilaterally ovariectomized as described previously (Fleischer et al., 2021; Koss, Haertel, Philippi, & Frick, 2018) and injected with AAV (or saline control) into the RE within the same surgical session. Ovariectomy eliminates the primary source of circulating estrogens, which affect spatial memory in mice (Frick & Berger-Sweeney, 2001).

Immediately after ovariectomy, pAAV8-CaMKIIa-HA-KORD-IRES-mCitrine (a gift from Bryan Roth, Duke Vector Core, titer 4.2x1012 vg/mL), AAV8-CaMKIIa-eGFP (Duke Vector Core, diluted in sterile saline to 4.2x1012 vg/mL), or sterile saline (sham control) was infused into the RE, resulting in 3 groups: 1) saline control, 2) GFP control, and 3) KORD. A 26-gauge Hamilton syringe with flat needle tip was secured within an infusion pump (Stoelting) mounted to the stereotaxic apparatus (Kopf) positioned to target the RE (AP: −0.6 mm; DV: −4.0 mm; ML: 0 mm relative to Bregma) at a 15-degree angle to avoid hitting the midline sinus. The syringe was left in place for 2 min and was then withdrawn 0.1 mm to create a pocket into which the virus could diffuse. Virus (or saline) was infused at a rate of 0.1 μL/min for 5 min (0.5 μL total), and the needle remained in place for 8 min after infusion to allow time for diffusion. The syringe tip was then slowly withdrawn to prevent liquid aspiration back into the syringe. The incision site was sutured and antibacterial ointment (Neosporin) applied to the wound. For analgesia, mice were given a subcutaneous injection of Rimadyl (1:100, 10 mg/kg) at the beginning of surgery, as well as one and two days post-op. Mice were given a minimum of three weeks before the onset of behavioral testing to allow for recovery and sufficient viral expression.

2.3. Drug administration

The KORD actuator salvinorin B (SALB; Cayman Chemical, Ann Arbor, MI) was prepared as described previously (Tuscher et al., 2018). SALB was dissolved in 100% DMSO (Fisher Scientific, Pittsburg, PA) to a concentration of 10 mg/mL, aliquoted, and stored at −20 °C. On the day of injection, an aliquot was thawed, briefly warmed to 40 °C, and vortexed to ensure that the compound was fully dissolved. SALB was injected intraperitoneally (i.p.) at a dose of 10 mg/kg. Post-training i.p. injection of this SALB dose impairs object placement memory consolidation in ovariectomized mice expressing the KORD in the dorsal hippocampus (Tuscher et al., 2018). Mice were weighed 1–2 days prior to behavioral testing to avoid additional stress on the testing day.

2.4. Object placement testing

Training and testing were conducted in a white open field box (60 × 60 × 47 cm) as described previously (Fleischer et al., 2021). To habituate mice to experimenter handling, they were first handled for 1 min/day for 3 days. For two subsequent days, mice were habituated to the empty open field box and allowed to roam freely for 5 min. During training, mice were exposed to two identical objects in the northeast and northwest corners of the box and were given 20 min to accumulate 30 sec exploring the objects. Object exploration was defined as direct contact between the nose and the object. Treatments were administered either 10 min prior to training or immediately after training. For pre-training injections, mice were injected with saline or SALB 10 min prior to training. For post-training treatments, mice were removed from the box immediately after accumulating 30 sec of exploration, and were then injected with saline or SALB to target the memory consolidation

process and returned to their home cages.

Memory was tested 4 h later, a time point at which vehicle-treated mice remember the location of the training objects (Tuscher et al., 2018). During testing, one training object was moved to the southeast or southwest corner of the testing box. Again, mice remained in the box until they accumulated 30 sec exploring the objects. If a mouse did not explore for 30 sec within 20 min, then the trial ended and the mouse was re-trained at least one day later with different objects.

Any mouse that did not explore for at least 27 sec during training or 30 sec during testing was excluded from data analysis. Time spent with the objects, time to accumulate 30 sec of exploration, and path length were recorded using AnyMaze software (Stoelting). Because mice inherently prefer novelty, those who spent significantly more time with the moved object than chance (15 sec) were considered to have intact memory for the locations of the original training objects.

2.5. Tissue collection and immunohistochemistry

Tissue was collected a minimum of one week after the completion of object placement testing. To study the effects of KORD-mediated inactivation on neuronal activity in the RE, mice were trained with objects as described above and tissue collected 1 h later to quantify levels of the immediate early gene EGR-1. One hour was selected because previous studies have reported that EGR-1 is elevated in the dorsal hippocampus 1 h after fear conditioning training in male rats (Huckleberry et al., 2015; Lonergan, Gafford, Jarome, & Helmstetter, 2010). Mice who did not accumulate at least 15 sec of exploration within the 20-min session were excluded from the study.

Twenty mice who previously received pre-training SALB injections were injected with SALB 10 min prior to being exposed to two identical novel objects and were then given up to 20 min to accumulate 30 sec of exploration. Immediately after training, mice were returned to their home cage for 60 min. They were then deeply anaesthetized using a cotton ball soaked with isoflurane, and transcardially perfused with ice cold 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). Mice assigned to the homecage control group were not injected or trained before perfusion. Brains were post-fixed overnight in 4% PFA in PBS, then placed in a 30% sucrose solution for a minimum of 3 days or until they had sunk to the bottom of the vial. Brains were blotted to remove excess sucrose, flash-frozen on dry ice, sliced using a cryostat at −20 °C, and mounted onto Superfrost Plus slides (Fisher Scientific) or stored at −20 °C in a cryoprotectant solution until they were processed for immunohistochemistry (IHC). Three mice were excluded from analysis: one in the KORD group with off-target viral expression, and two (one KORD, one saline sham) that did not accumulate at least 15 s of object exploration during training.

To verify virus placement, one in every six sections of the RE (20 μM thickness) from eGFP-expressing mice was mounted to a slide, coverslipped with aqueous mounting medium containing DAPI (Santa Cruz), and imaged under a fluorescent microscope. Tissue from mice expressing KORD was immunostained for the HA-epitope tag on the KORD receptor. Sections were treated with 1% sodium borohydride ($NaBH_4$) for 15 min, and then rinsed 2x5 mins in PBS, blocked in 10% normal goat serum (NGS, Biogenex) and incubated in HA-tag primary antibody (1:1000, Cell Signaling #3724) overnight. The next day, tissue was washed 3x5 minutes and incubated in Alexa-Fluor 594 goat anti-rabbit IgG secondary (1:500, ThermoFisher #A-11012) to allow co-visualization of the HA tag with the mCitrine fluorophore. Viral expression was viewed with a Nikon fluorescent microscope. For saline controls, one in every six sections was stained with cresyl violet and imaged under a brightfield microscope to check for tissue damage made by the syringe during surgery. Placements were localized with the aid of a mouse brain atlas (Paxinos & Franklin, 2001).

To label EGR-1 expression, three sections of tissue containing RE between −0.4 mm and −0.96 mm relative to Bregma were washed in 25 mM PBS to remove cryoprotectant, blocked with 5% NGS in 25 mM PBS with 0.3% TritonX-100, and incubated overnight at 4 °C in EGR-1 rabbit primary antibody (1:500 in block solution, Cell Signaling #4153). The next day, tissue was washed 3x5 min, incubated in Alexa-Fluor 594 goat anti-rabbit IgG secondary (1:500 in 0.3% TritonX-100 in PBS) for 90 min at room temperature, washed 3x5 min, and mounted and coverslipped with aqueous DAPI mounting medium (Santa Cruz). Tissue was protected from light at all times to avoid photobleaching of the mCitrine tag.

2.6. Image acquisition and analysis

Z-stack images (15 slices per stack, step size 1.22 μm) were acquired on a confocal microscope (Olympus Fluoview FV1200) at 20× magnification, saved as a 12-bit TIFF, and analyzed with FIJI software (open source ImageJ; (Schindelin et al., 2012)). For each brain region, three tissue slices were quantified, and the average used for analysis. In FIJI, images were quantified using previously established methods (Ferrara, Trask, Pullins, & Helmstetter, 2019). Briefly, images were Gaussian filtered (sigmas of 2 and 1.5), thresholded using the Triangle method, and a count of all particles 4 or greater in diameter was conducted using watershed segmentation to isolate touching particles. Particle count for each region and treatment condition was expressed as a % of homecage control. Data were coded such that image processing in FIJI and subsequent analysis were conducted by an experimenter blind to treatment condition.

2.7. Statistical analysis

Prism 8 (GraphPad) was used for all statistical analyses. To assess learning within each group, OP data were analyzed using one-sample *t*-tests to determine if the time spent with each object differed significantly from chance (15 sec). In all analyses in which GFP and sham groups did not differ significantly from each other (as determined by two-tailed *t*-tests), groups were merged into a single control group. To determine effects of KORD expression on memory relative to controls, unpaired two-tailed *t*-tests were conducted on time spent with the novel and moved objects. Time to accumulate 30 sec of exploration and IHC data were also analyzed using two-tailed *t*-tests. Significance was set at $p < 0.05$ for all tests.

3. Results

3.1. AAV expression

Representative images depicting the spread and expression of AAVs are displayed in Fig. 1. In all mice, the eGFP and KORD AAVs were expressed in the RE and were largely restricted to the RE. The extent of spread and signal intensity of AAV8-CaMKIIa-eGFP was generally greater than that of pAAV8-CaMKIIa-HA-KORD-IRES-mCitrine, which is likely a result of differences in construct size.

3.2. Inactivation of the RE prior to training impairs spatial memory acquisition

To determine whether the RE is necessary for OP memory acquisition, ovariectomized mice were infused into the RE with saline (n = 6), GFP-AAV (n = 5), or KORD-AAV (n = 13) at least three weeks prior to training to allow sufficient time for optimal virus expression (Fig. 2 A-B). Mice received an i.p. injection of SALB (10 mg/kg) 10 min before the start of object training. As in our previous work using inhibitory DREADDs (Tuscher et al., 2018), OP was tested 4 h later. Because no measure of performance in the OP task differed between saline and GFP-AAV controls, these groups were combined into a single control group. Mice in the control group spent significantly more time than chance (15 sec) with the moved object ($t_{(9)} = 3.396$, $p = 0.0079$; Fig. 2C), whereas the KORD group did not ($t_{(10)} = 0.7269$, $p = 0.484$; Fig. 2C). Moreover,

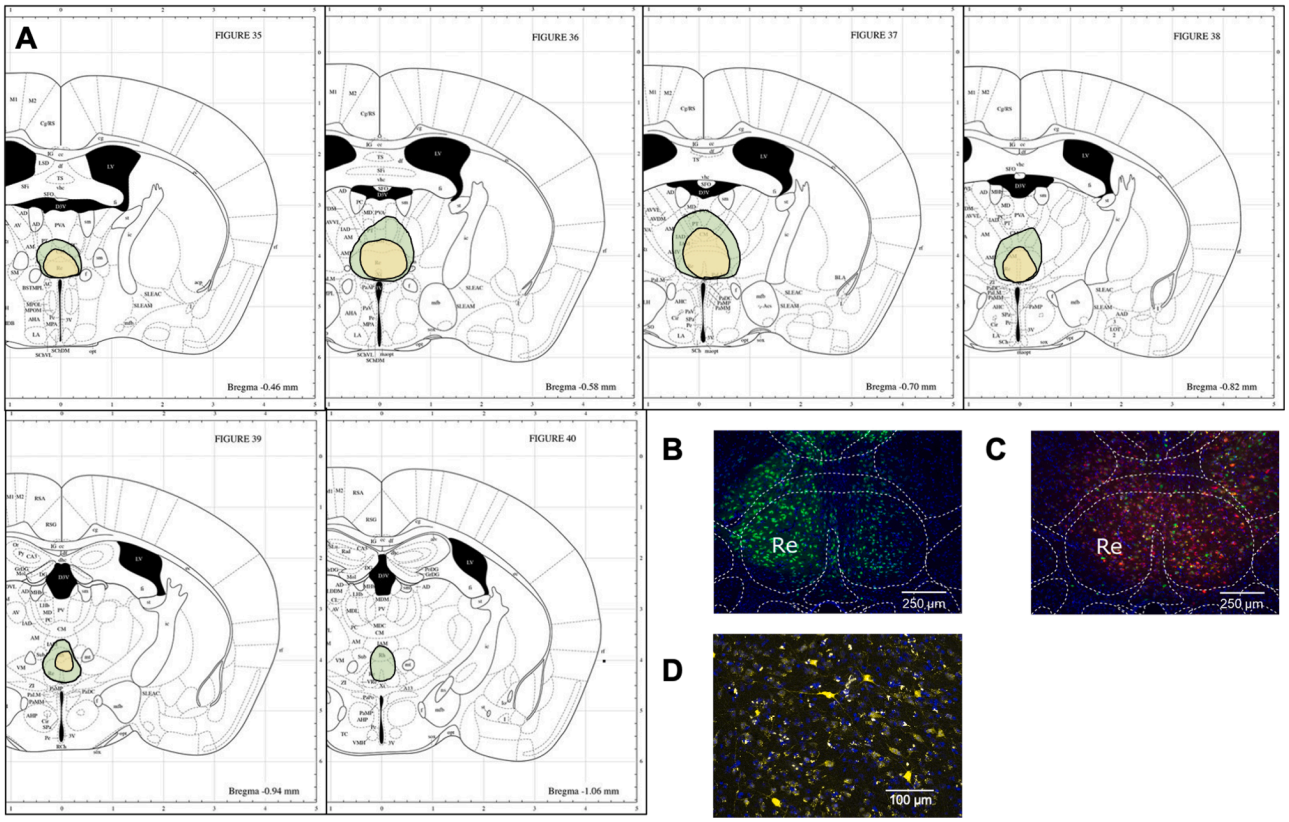

Fig. 1. Representative Images Depicting AAV Spread and Expression. (A) Representative spread of the eGFP (Green) and mCitrine (Yellow) fluorophore tags. (B-D) Representative expression of eGFP (B) and mCitrine in green and HA-tag in red at 10x (C) and mCitrine in yellow at 20x (D). For panels B-D, the approximate Paxinos and Franklin (2001) plate # is 37, which is −0.70 mm relative to Bregma. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the KORD group spent significantly less time with the moved object than the control group, as determined by an unpaired, two-tailed *t*-test ($t_{(19)} = 2.309$, $p = 0.0323$; Fig. 2C). Combined, these data suggest that that RE inactivation prior to training impaired spatial memory acquisition.

To ensure that this impairment was not due to an effect of RE inactivation on motivation to explore or mobility, time to accumulate 30 sec of exploration and path length were analyzed using unpaired *t*-tests. The control and KORD groups did not differ in the path length traveled during exploration ($t_{(19)} = 1.897$, $p = 0.0731$; Fig. 2D) or the time to accumulate 30 sec exploration ($t_{(19)} = 0.9134$, $p = 0.3725$; Fig. 2E), indicating that RE inactivation did not affect exploratory motivation or ability.

3.3. RE inactivation immediately after training impairs spatial memory consolidation

Next, a new set of mice [saline (n = 6), GFP-AAV (n = 6), or KORD-AAV (n = 12)] received an i.p. injection of SALB (10 mg/kg) immediately after completion of object training to restrict RE inactivation to the early consolidation phase of memory (Fig. 3A-B). Similar to the pre-training experiment, control mice tested in OP spent significantly more time than chance with the moved object ($t_{(8)} = 4.413$, $p = 0.0022$; Fig. 3C), whereas the KORD group did not ($t_{(8)} = 0.6067$, $p = 0.5609$; Fig. 3C), demonstrating impaired spatial memory consolidation. An unpaired *t*-test suggested a trend towards an impairment in the KORD group relative to controls ($t_{(16)} = 1.945$, $p = 0.0696$). As in the pre-training experiment, there were no significant between-group differences in path length ($t_{(16)} = 1.172$, $p = 0.2583$, Fig. 3D) or time to accumulate 30 sec exploration ($t_{(16)} = 1.782$, $p = 0.0937$, Fig. 3E).

3.4. KORD inactivation reduces EGR-1 expression

Lastly, we investigated whether RE inactivation reduces training-induced elevations in neuronal activity. Three weeks after the completion of behavioral testing, mice from the pre-training experiment were again injected with 10 mg/kg SALB 10 min prior to training and then explored two identical novel objects for 30 sec. They were then perfused 1 h later. Protein levels of the immediate early gene EGR-1 were then measured in the RE via fluorescent IHC (Fig. 4A-C). Mice in the KORD group exhibited reduced EGR-1 expression relative to the combined control group ($t_{(14)} = 3.489$, $p = 0.0036$; Fig. 4D), suggesting that activation of KORD receptors reduced neuronal activity in the RE.

4. Discussion

The primary goal of this study was to determine the extent to which activation of excitatory neurons in the RE are necessary for OP memory acquisition and consolidation. On the basis of previous evidence suggesting a role for the RE in mediating hippocampus- and mPFC-dependent memories (Jung et al., 2019; Layfield et al., 2015; Vetere et al., 2017), we hypothesized that inactivation of excitatory RE neurons would block acquisition and consolidation of memory for object location in this DH- and mPFC-dependent task (Boulware, Heisler, & Frick, 2013; Tuscher et al., 2018, 2019). To test this hypothesis, we used the KOR-DREADD to reversibly inactivate the RE during the acquisition and consolidation phases of memory formation. We found that chemogenetic inactivation of the RE prior to or immediately after training impaired acquisition and consolidation of memory in the OP task compared to controls. Importantly, inactivation of RE during training did not affect exploratory behaviors, including motivation and ability to explore. We also showed that RE inactivation reduces EGR-1 expression

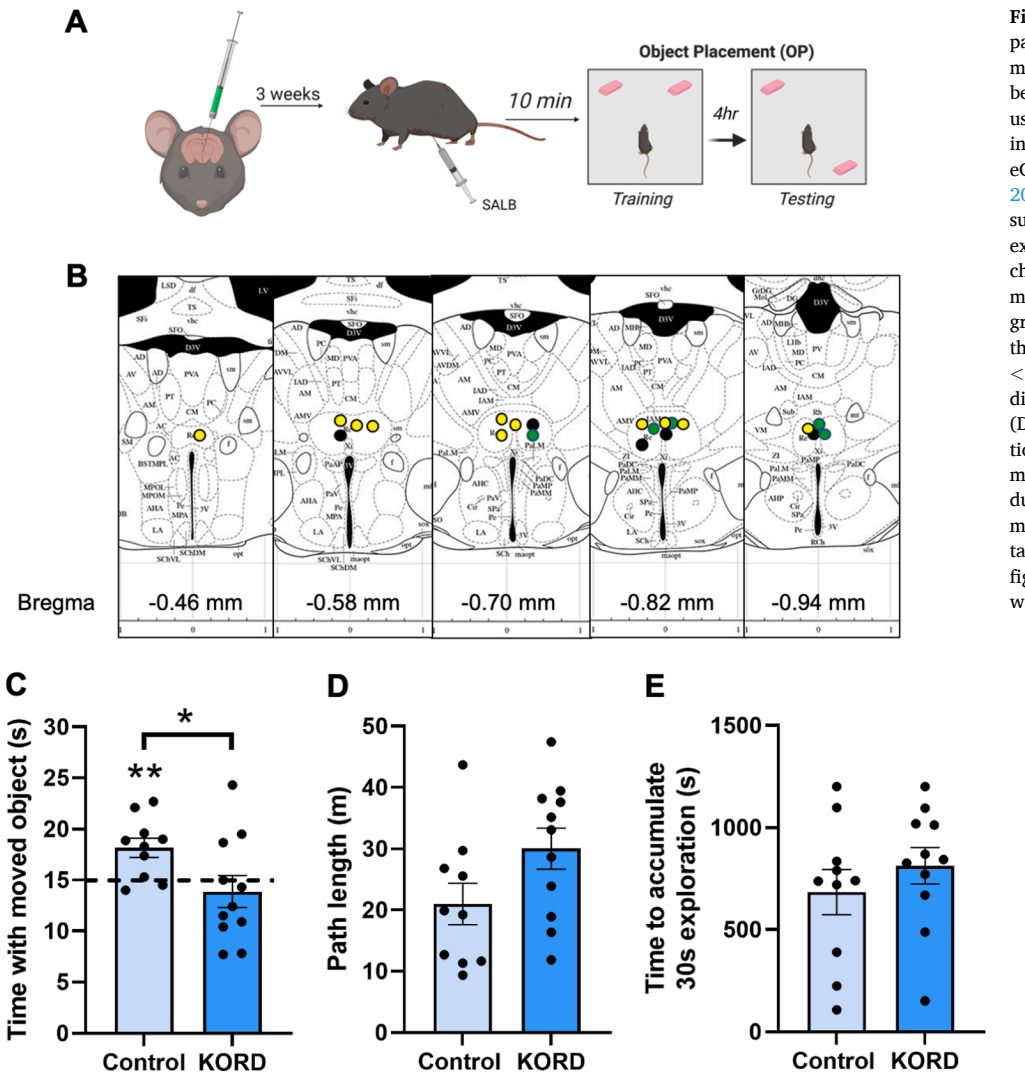

Fig. 2. Pre-training inactivation of RE impairs spatial memory acquisition. (A) Schematic of AAV infusion, SALB injection, and behavioral testing timeline. Panel created using BioRender.com. (B) Locus of AAV infusion sites (yellow = KORD, green = eGFP, black = saline; Paxinos & Franklin, 2001). Each circle represents an individual subject. (C) Control mice, but not mice expressing the KORD, spent more time than chance (dashed line at 15 sec) with the moved object (**$p < 0.01$). The control group also spent significantly more time with the moved object than the KORD group (*$p < 0.05$). Control and KORD mice did not differ significantly in exploration path length (D) or the time to accumulate 30 s exploration (E) during OP training ($p > 0.05$ for both measures), indicating that inactivation of RE during the training phase did not affect non-mnemonic aspects of the task. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the RE, confirming reduction of neural activity in the RE by the KORD manipulation. Taken together, these data suggest that the RE plays a role in spatial memory among female mice, particularly during acquisition and the early consolidation periods.

To the best of our knowledge, this study is the first to assess the effects of reversible RE inactivation on OP memory in rodents of either sex or to examine RE function in females. Our effects indicate a role for the RE in both OP acquisition and consolidation, suggesting that multiple phases of OP memory formation depend on RE activity. This finding is consistent with a previous study in which electrolytic lesion of the RE in male mice impaired preference for the moved object during the second half of a 5-minute testing trial (Jung et al., 2019). The fact that RE inactivation impaired OP memory in both male (Jung et al., 2019) and female (present study) mice suggests a similar role for the RE in mediating spatial memory in both sexes. However, another study reported no effect on OP memory of excitotoxic NMDA lesions in male rats (Barker & Warburton, 2018). Potential reasons underlying this discrepancy are unclear because there are so few studies on the effects of RE inactivation in OP memory, each with their own protocols, parameters, and methods of inactivation. Nevertheless, the present data suggest a potentially important contribution of the RE to OP memory formation that warrants further investigation.

Additionally, our OP findings are consistent with literature using reversible RE inactivation reporting a role for the RE in other tasks that rely on both the mPFC and hippocampus. In particular, the RE appears necessary for contextual memory, as illustrated in studies using contextual fear conditioning. For example, muscimol inactivation before acquisition or retrieval impaired contextual fear conditioning (Ramanathan et al., 2018), and the acquisition, but not retrieval, of trace fear conditioning (Lin, Chiou, & Chang, 2020) in male rats. Further, chemogenetic inactivation using hM4Di also impaired contextual fear conditioning in male mice (Vetere et al., 2017). Thus, the RE appears to regulate multiple forms of hippocampus- and mPFC-dependent memory.

However, this conclusion is complicated by inconsistencies among studies examining a role for the RE in other spatial memory tasks, each of which measure different aspects of spatial memory. For example, in one study using the Morris Water Maze (MWM), tetracaine-induced inactivation of the RE during acquisition impaired escape latency, and inactivation immediately after acquisition or before a probe trial reduced time spent in target quadrant during the probe trial, suggesting that the RE is involved in each stage of spatial memory formation (Davoodi, Motamedi, Naghdi, & Akbari, 2009). However, ibotenic acid lesions of RE in rats influenced swimming strategy during a MWM probe trial, but did not affect overall learning of the platform location during acquisition (Dolleman-van der Weel, Morris, & Witter, 2009). Likewise, NMDA-induced lesions of RE in rats did not impair acquisition or memory in a probe trial given 5 days after acquisition, but did impair memory when the delay between acquisition and probe trial was 25 days (Loureiro et al., 2012). To add to the disparate results, muscimol-induced RE inactivation in rats during the testing phase of a

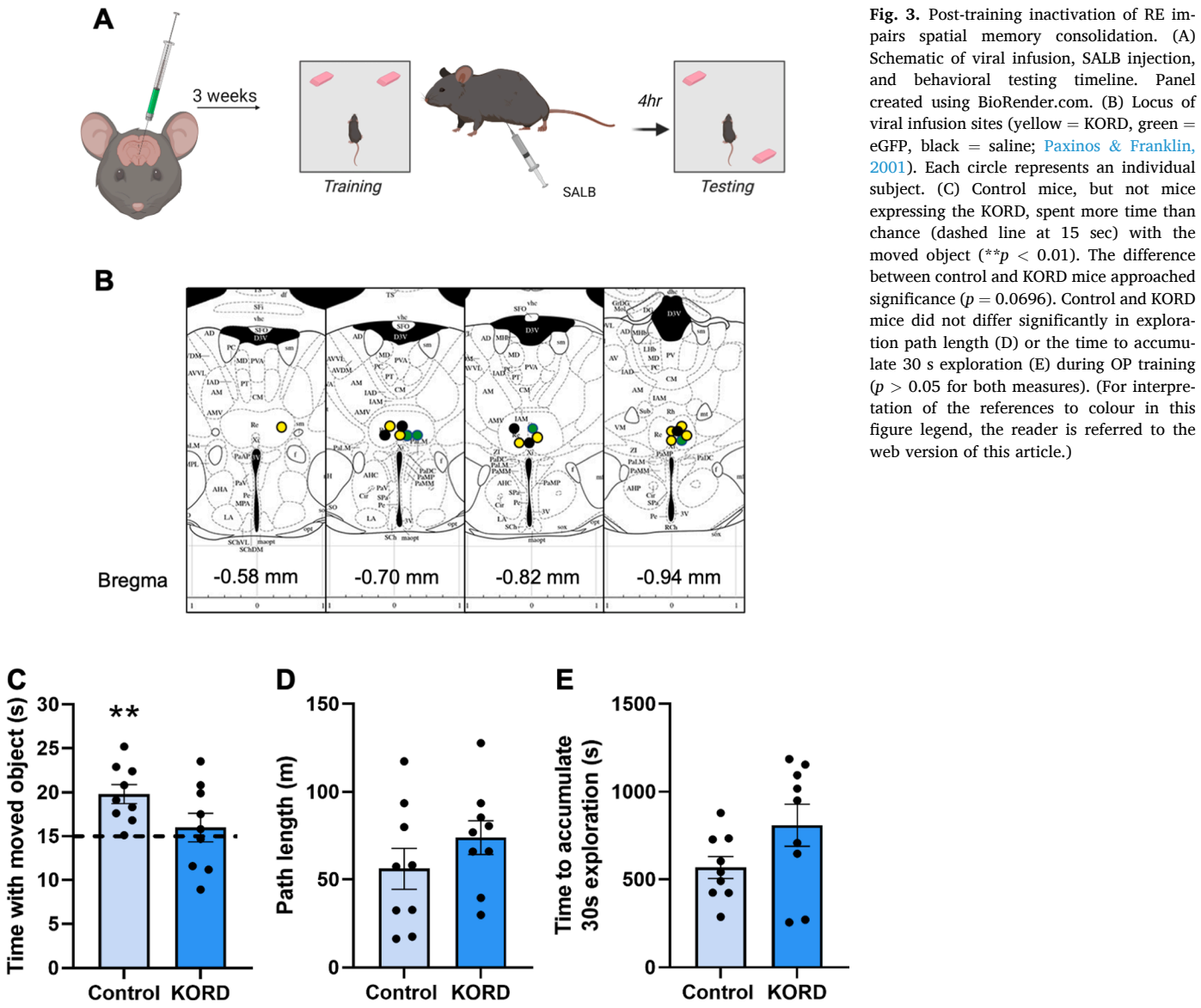

Fig. 3. Post-training inactivation of RE impairs spatial memory consolidation. (A) Schematic of viral infusion, SALB injection, and behavioral testing timeline. Panel created using BioRender.com. (B) Locus of viral infusion sites (yellow = KORD, green = eGFP, black = saline; Paxinos & Franklin, 2001). Each circle represents an individual subject. (C) Control mice, but not mice expressing the KORD, spent more time than chance (dashed line at 15 sec) with the moved object (**$p < 0.01$). The difference between control and KORD mice approached significance ($p = 0.0696$). Control and KORD mice did not differ significantly in exploration path length (D) or the time to accumulate 30 s exploration (E) during OP training ($p > 0.05$ for both measures). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

crossword-like maze impaired cue-based spatial navigation ability, whereas inactivation after each daily training session (targeting the consolidation phase) did not affect memory (Mei, Logothetis, & Eschenko, 2018). These discrepant findings could result from several methodological factors, including inactivation method and differences in task protocols, task complexity, duration of training, length between training and probe testing, and other factors unique to individual laboratories. In particular, the method of inactivation (lesioning vs. pharmacological inactivation) could play an important role. Permanent lesions take one brain region "offline" while allowing other regions to compensate for the loss of function, thereby fundamentally changing brain connectivity in a way that can influence all aspects of memory formation. In contrast, pharmacological or viral-mediated inactivation transiently and reversibly perturb an intact system at a distinct period during the memory formation process. As such, the fundamental impacts of lesion- and DREADD-based RE silencing are quite different, so it is perhaps not surprising that their effects on memory differ. Taken together, the results of these studies suggest that the method of inactivation and task parameters may be important factors to consider when investigating the RE's role in spatial memory. Nevertheless, based on previous evidence that OP requires activity of other regions that are well-connected to RE, namely the DH and prelimbic/infralimbic region of the mPFC (Tuscher et al., 2018), we believe that the OP task is sufficient to test the role of RE in episodic-like spatial memory.

One final potential issue is whether ovariectomy surgery may have influenced the results. However, it is highly unlikely that the role of the RE in spatial memory acquisition and consolidation would differ based on gonadal status. Sex steroid hormones such as the potent estrogen 17β-estradiol modulate the function of brain regions but do not fundamentally alter their nature. Thus, estrogens produced by the ovaries can alter the strength of a memory, such that it lasts longer or is more resistant to extinction or reconsolidation, but do not influence the types of memory mediated by a brain region. For example, our laboratory has shown repeatedly that infusion of 17β-estradiol into the dorsal hippocampus of ovariectomized mice or gonadectomized male mice makes object recognition and object location memories last longer than in mice treated with vehicle (Gross, Alf, Polzin, & Frick, 2021; Koss et al., 2018; Tuscher, Taxier, Schalk, Haertel, & Frick, 2019). However, our work demonstrates that the function of the dorsal hippocampus in mediating these types of memories is the same, regardless of whether or not the mice have gonads or are treated with exogenous hormones (Gross et al., 2021; Koss et al., 2018; Tuscher et al., 2019). As such, we expect that the role of the RE in mediating spatial memory would have been the same in intact females and among intact or gonadectomized males.

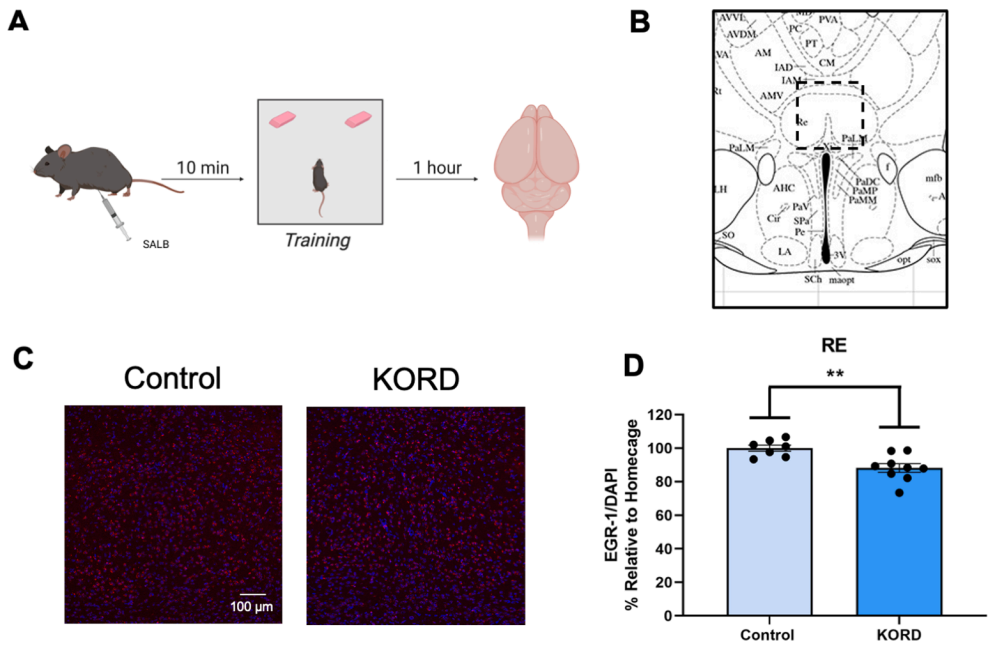

Fig. 4. KORD-mediated inactivation decreased EGR-1 expression in RE. (A) Schematic of SALB injection, object training, and tissue collection. Panel created using BioRender.com. (B) Representative area of EGR-1 quantification (Paxinos & Franklin, 2001). (C) Representative EGR-1 expression (red) against DAPI (blue) in RE in Control and KORD groups, 20x magnification. (D) Treatment with SALB 10 min prior to training decreased EGR-1 expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In conclusion, thei present study suggests that the RE is essential for the acquisition and consolidation of spatial object memories in female mice. As such, it may play a role in mediating communication between the DH and mPFC, given that it maintains reciprocal connections to these brain regions that also mediate object placement memory (Boulware et al., 2013; Jung et al., 2019; Tuscher et al., 2018, 2019). Future work will address this possibility using projection-specific chemogenetic inactivation. Additionally, this study provides the first evidence for a role of the RE in memory formation in females, suggesting similar importance of this thalamic region in spatial memory for both sexes. Thus, the present data provide important new insights about the involvement of the RE in spatial memory formation that may have important implications for elucidating the neural circuitry of episodic memory formation in both males and females.

CRediT authorship contribution statement

Miranda R. Schwabe: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Carnita M. Lincoln:** Formal analysis, Investigation. **Margaret M. Ivers:** Investigation. **Karyn M. Frick:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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