

INACTIVATION OF NUCLEUS REUNIENS IMPAIRS SPATIAL MEMORY IN MICE

by

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ABSTRACT

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Episodic memory is a complex process requiring input from several regions of the brain. Coordinated activity in the Dorsal Hippocampus (DH) and medial Prefrontal Cortex (mPFC) is required for episodic memory consolidation. Our laboratory demonstrated that simultaneous subthreshold chemogenetic inactivation of the dorsal hippocampus (DH) and medial prefrontal cortex (mPFC) impairs the consolidation of object placement (OP) and object recognition (OR) memory in female mice (Tuscher et al., 2018), suggesting that these two brain regions work in concert to promote 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 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 and fear extinction learning. However, it is not clear to what extent the RE is necessary for OR and OP memory.

The goal of this study was to determine whether activity in the RE is necessary for OP and OR memory. Kappa-opioid receptor DREADD (KORD) virus activated by salvinorin B was used to inactivate excitatory neurons in the RE. Mice infused with GFP virus or saline were used as controls. During training, mice were allowed to explore 2 identical objects placed near the corners of a large white box, and received a 10 mg/kg injection of salvinorin B either 10 minutes prior to training or immediately after training to target effects to the encoding and consolidation phases of memory, respectively. Testing was conducted 4h after training for OP or 24h for OR, timepoints at which control mice remember the location of training objects. During testing, one object was moved to a different quadrant of the testing box. Activation of the KORD prior to or immediately after training blocked OP memory relative to chance and controls. To determine the effect of RE inactivation on neuronal activity, expression of the immediate early gene EGR-1 was measured via fluorescent immunohistochemistry 1 hr after an object training trial. Object training alone did not increase EGR-1 expression relative to homecage controls, but KORD activation of RE resulted in a small but significant decrease in EGR-1 expression. In summary, the findings of this study support a key role for the RE in spatial memory learning and consolidation.

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LIST OF ABBREVIATIONS

DH	Dorsal Hippocampus
DREADD	Designer Receptor Exclusively Activated by Designer Drug
EGR-1	Early Growth Response protein 1
IL	Infralimbic Cortex
KORD	Kappa Opioid Receptor DREADD
mPFC	medial Prefrontal Cortex
OP	Object Placement
OR	Object Recognition
PL	Prelimbic Cortex
RE	Nucleus Reuniens of the Thalamus
VH	Ventral Hippocampus

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INTRODUCTION

The ability to remember is often taken for granted but is essential for most aspects of daily life. Memory loss, a common symptom of neurodegenerative disease, mental health disorders, and aging, robs people of their independence and diminishes their quality of life. One type of memory particularly vulnerable to loss is episodic memory, a type of declarative memory which binds sensory stimuli and contextual information into a rich narrative form (Allen & Fortin, 2013; Dickerson & Eichenbaum, 2010). Episodic memory is critical for an individual's sense of identity, ability to participate in society, and overall wellbeing. As such, there is a pressing need to identify the molecular and cellular mechanisms in the brain facilitating memory consolidation. This knowledge may be leveraged to enable development of the next generation of therapies that can reduce memory dysfunction in neurodevelopmental disorders, psychiatric illnesses, and neurodegenerative diseases.

Dorsal Hippocampal-Prefrontal Cortex Interactions in Episodic Memory

The acquisition, consolidation, and retrieval of episodic memories are complex processes that involve combining multiple modes of sensory stimuli with context and temporal cues. These integrated memories are stored as "episodes" that serve as the basis of personal narrative for one's life. The hippocampus is integral for binding information across sensory modalities and is one of the most thoroughly studied brain regions involved in memory (Eichenbaum, 2000; Lavenex & Amaral, 2000; Squire, 1992). Decades of work utilizing lesions or pharmacological inactivation has demonstrated an essential role for the hippocampus in many forms of learning and memory, leading some to posit that the hippocampus is the primary region responsible for episodic memory

(Squire, 1992; Squire & Alvarez, 1995). Early evidence from amnesic patients, nonhuman primates, and rodents pointed to a unique role of the hippocampus and adjacent medial temporal lobe areas in spatial and certain nonspatial components of memory (Eichenbaum et al., 1990; Zola et al., 2000). The rodent hippocampus has been functionally divided into a dorsal portion (DH) and ventral portion (VH); the DH is thought to mediate spatial and contextual memories, whereas the VH is involved in emotional memories and stress (Fanselow & Dong, 2010). Because this thesis focuses on spatial and object recognition memory, only the DH will be discussed below.

More recent research has revealed that episodic memory requires coordinated activity, either concurrent or sequential, among many brain regions in addition to the dorsal hippocampus. One key partner in mediating episodic memory 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 et al., 2017; Jin & Maren, 2015; Jones & Wilson, 2005; Kitamura et al., 2017; Warburton & Brown, 2015). These findings were historically achieved by lesioning or pharmacologically inactivating regions to observe an impairment (reviewed in Warburton and Brown, 2015) or recording synchronous activity between the two regions during a spatial memory task (Jones & Wilson, 2005). More recent studies have used sophisticated methods like chemogenetic inhibition (Maharjan et al., 2018) or genetically targeted tetanus toxin inactivation of DH-mPFC projections activated during memory formation (Kitamura et al., 2017). For example, previous research in the Frick laboratory (Tuscher et al., 2018; see “Manipulating Neuronal Activity with DREADDs”

section below) found that chemogenetic inactivation of the mPFC or DH in ovariectomized female mice impaired consolidation of object recognition and object placement memory (Tuscher et al., 2018). Furthermore, simultaneous subthreshold inactivation of mPFC and DH, that is, at doses that did not impair memory on their own, impaired memory consolidation on these tasks. These data suggest that concurrent activity in these two regions is necessary for object memory consolidation (Tuscher et al., 2018).

Despite the demonstrated importance of DH-mPFC interactions for memory consolidation, the nature of the connection between the DH and mPFC is still not fully understood. Compared to the input from VH, there are relatively few direct projections from the CA1 and subiculum of DH to layers 1, 5 and 6 of the PL and IL regions of the mPFC (Jay et al., 1992; Verwer et al., 1997) (See Fig. 1). The density of these projections increases along the longitudinal axis of the hippocampus from dorsal to ventral (T. M. Jay & Witter, 1991; Verwer et al., 1997) and are involved in contextual fear memory (Ye et al., 2017). 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 projections have been identified from IL or PL to the DH. Due to the limited nature of direct connections between the DH and mPFC, it is believed that the majority of communication from the mPFC to DH related to episodic memory occurs indirectly through other regions such as the entorhinal cortex, perirhinal cortex, and nucleus reuniens of the thalamus (RE) (Chao et al., 2020; Jin & Maren, 2015). The RE was chosen for the focus of this thesis because of its substantial bidirectional connections to both DH and mPFC and the relative lack of data on RE's role in hippocampally-involved memory.

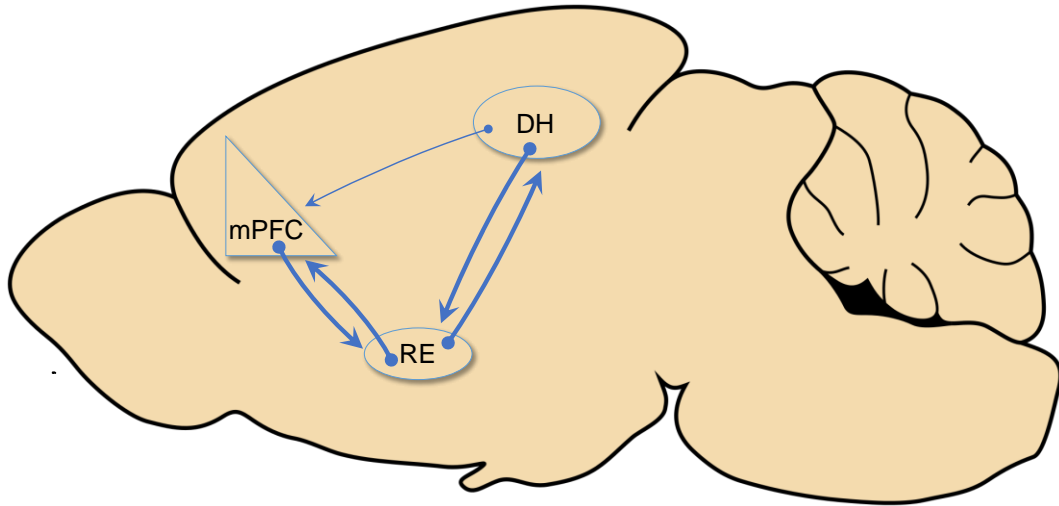


Figure 1. Diagram of projections among the dorsal hippocampus, nucleus reuniens, and medial prefrontal cortex. Dashed line indicates less dense but behaviorally relevant connection. DH = dorsal hippocampus, mPFC = medial prefrontal cortex, RE = nucleus reuniens of the thalamus.

The Nucleus Reuniens –Connectivity and Cytoarchitecture

The Nucleus Reuniens is a small, ventral midline thalamic nucleus. In rodents, the RE is located directly above the ventral 3rd ventricle, separated from the ventricle only by the xiphoid nucleus (Assini et al., 2009; Barrett et al., 2011). The RE is notably well-connected to several other brain regions involved in episodic memory. It is bidirectionally connected to the mPFC and hippocampus (Figure 1: Hoover & Vertes, 2007; McKenna & Vertes, 2004; Varela et al., 2014; Vertes, 2004) and sends unidirectional projections to the perirhinal and entorhinal cortices (Wouterlood, 1991). Within the hippocampus, the RE sends projections to apical dendrites in the stratum lacunosum moleculare of the CA1 region, inhibitory interneurons in the strata oriens/alveus and radiatum (Dolleman-Van der Weel et al., 1997; Dolleman-Van der Weel & Witter, 2000), and the molecular layer of the subiculum (Vertes et al., 2006). The number of projections to VH are an estimated 10x greater than the number in DH (Hoover & Vertes, 2012). The RE and peri-RE, a region structurally and functionally connected to the RE and which surrounds the RE on

the top and sides, send projections to layers 1 and 5/6 of the mPFC (Hoover & Vertes, 2012; Varela et al., 2014). A small number of RE neurons project to both hippocampus and mPFC (Hoover & Vertes, 2012). The nature of these projections may allow for bidirectional indirect communication between mPFC and DH mediated by the RE, by forming a complete circuit from the DH to mPFC, and back to the DH through the RE (Vertes et al., 2007) (See Fig. 1).

The excitatory projections originating in the RE use glutamate or aspartate as their primary neurotransmitter (Bokor et al., 2002; Cruikshank et al., 2012). Populations of calretinin- and calbindin- containing cells have been identified suggesting the presence of inhibitory cells (Arai et al., 1994; Bokor et al., 2002), however the functional significance of these populations are not fully understood. Recent research in this area found that calretinin-positive projections from RE to hippocampus were differentially active in theta oscillations and sharp wave ripples (recorded in DH) compared to calretinin-absent projections, regardless of the presence of calbindin (Lara-Vásquez et al., 2016). Interestingly, neurons that project to both mPFC and dorsal or ventral hippocampus do not express calretinin or calbindin but are surrounded by populations of calretinin or calbindin expressing cells (Viena et al., 2020). Thus, thus the precise nature of these projection neurons remains unclear. They are unlikely to express parvalbumin or somatostatin, as these proteins are not found in RE cells (Bokor et al., 2002; Viena et al., 2020).

A Role for the Nucleus Reuniens in Memory

The RE appears to be necessary for cognitive functions that involve coordination between the mPFC and dorsal and/or ventral hippocampus. One such function that

requires extensive and simultaneous coordination between these two regions is spatial working memory, in which rodents must integrate spatial cues and direction (DH) and the executive function of working memory (mPFC) in order to make accurate navigation decisions (Griffin, 2015). In the spatial alternation T-maze, a task commonly used to assess spatial working memory, rodents are placed in a figure-8 maze and must make alternating left and right turns to obtain a food reward. To correctly select the arm with food, subjects must both remember their previous arm selection and use external spatial cues to navigate through the maze. Task difficulty can be increased by inserting a several second delay between trials, thereby requiring the memory of the previous trial to be kept “in mind” during the delay. In male Long-Evans rats, pharmacological inactivation of the RE via the GABA_A agonist muscimol impaired choice accuracy when there was a 5- or 30- second delay, but not when there was no delay, indicating a role for RE in spatial working memory (Layfield et al., 2015). Using optogenetics, Maisson et al. (2018) found that inactivation of RE during the sample phase, but not delay or choice phase, impaired choice accuracy on the spatial alternation T-maze, suggesting involvement of the RE in the encoding of spatial working memories (Maisson et al., 2018). The RE is thought to contribute to spatial working memory by facilitating collaborative timing between the mPFC and DH known as oscillatory synchrony, as inactivating RE disrupts this oscillatory synchrony (Hallock et al., 2016). Similar effects of RE inactivation have been observed in other studies of synchrony, for example, in the slow oscillation activity during sleep that is thought to be necessary for long-term memory consolidation (Ferraris et al., 2018; Hauer et al., 2019).

The RE's contributions to memory in other tasks have been investigated as well. In mice, the RE was identified as critical among a network of brain regions that facilitate contextual fear memory, as chemogenetic inactivation with hM4Di immediately after contextual fear training impaired freezing during testing (Vetere et al., 2017). Another study in mice observed increased synaptic strength at RE-mPFC and RE-CA1 synapses following trace eyeblink conditioning, a model of hippocampal-dependent associative learning (Eleore et al., 2011). Additionally, disrupting RE activity with high-frequency stimulation prior to the training phase impaired object recognition memory (Eleore et al., 2011). Permanent lesions of RE have had mixed results on spatial memory, with one study finding impairment on an object placement task (Jung et al., 2019) and another reporting impaired 24-hour memory in an object-in-place associative task but not a traditional object placement task (Barker & Warburton, 2018).

Studies investigating a role for the RE in memory have focused largely on male rats, mostly likely due to the tendency to use rats for complex behavioral tasks and the field's hesitance to incorporate female subjects into the research. Some published studies have investigated RE contributions to learning and memory in mice (Eleore et al., 2011; Jung et al., 2019; Vetere et al., 2017), but these studies used males only. Therefore, not much is known about the RE's role in mouse models of memory and even less about whether sex differences exist in the role of the RE in memory.

In summary, a growing body of data suggests a key role for the RE in memory functions involving both the mPFC and DH, particularly spatial memory. However, existing data have primarily been collected in male rats using permanent lesion methods. Such methods lack cellular and temporal specificity and preclude repeated assessment of RE's

role in memory consolidation. Reversible genetic inactivation methods allow specific cell populations (e.g., excitatory neurons) to be temporarily silenced, thereby providing both cellular and temporal specificity. However, there is a lack of data on RE in spatial and object recognition memory using a reversible method of brain inactivation. Thus, this thesis aims to address that knowledge gap by employing a reversible and specific method of neuronal inactivation – Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) – to test a role for the RE in object recognition and spatial memory consolidation.

Manipulating Neuronal Activity with DREADDs

To reversibly inactivate the RE and assess its role in memory acquisition and consolidation, we used DREADDs, a chemogenetic technique for manipulating neuronal activity in which mutated “designer” receptors are delivered to neurons via adeno-associated viral vectors (for review, see (Sternson & Roth, 2014)). These receptors no longer respond to an endogenous ligand, but rather to a synthetic ligand that has little off-target effects. The original DREADDs, hM4Di and hM3Dq (altered forms of the human muscarinic receptor coupled to G_i and G_q), bind to the designer drug clozapine-n-oxide (CNO), which can be administered via injection with little effect on cells not expressing the DREADDs (Armbruster et al., 2007).

We chose to use a more recently developed inhibitory DREADD based on the kappa opioid receptor (KOR-DREADDD, or KORD). The chemogenetic actuator for KORD is Salvinorin B (SALB), a metabolite of the kappa-opioid receptor agonist Salvinorin A. SALB is pharmacologically inert at doses of 10 mg/kg or less, with weak action on kappa opioid receptors at higher concentrations (Ansonoff et al., 2006; Vardy

et al., 2015). The half-life of SALB is ~20 minutes, significantly less than that of CNO, and rapidly reaches the brain after intraperitoneal injection (Vardy et al., 2015). The KORD receptor is a G_i -coupled GPCR, and receptor activation in neurons decreases neuronal firing, intracellular Ca^{2+} concentration, and cAMP production (Vardy et al., 2015; Whissell et al., 2016). KORD was chosen for this project because our laboratory previously found that KORD-mediated inactivation of the DH impairs object recognition (OR) and object placement (OP) memory consolidation in ovariectomized female mice, whereas hM4Di-mediated inactivation of the DH failed to produce any memory impairment (Tuscher et al., 2018). Thus, we hypothesized that KORD-mediated inactivation of RE would be most likely method to produce memory impairment in the RE if the RE is necessary for OR and OP memory consolidation.

Goal of this Thesis

A growing body of research finds that interaction between the DH and mPFC is important for memory, but this interaction may be mediated by other regions including the RE. Recent evidence suggests that the RE is necessary for several types of hippocampal-dependent memory. However, none of these studies have investigated whether the RE mediates memory in female mice, nor have any studies using a reversible method of inactivation such as DREADDs. Thus, this thesis was designed to determine the extent to which RE regulates hippocampal memory consolidation using the inhibitory KORD to temporarily inactivate the RE immediately after training.

METHODS

Subjects

Female C57BL/6 mice ($n=50$, in two cohorts of 24 each and 2 for pilot viral expression) at 8-10 weeks old upon arrival (Taconic, Cambridge City, IN) were used for this project. 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 Guidelines or *Guide for the Care and Use of Laboratory Animals*.

General Experimental Design

Mice were ovariectomized and received an intracranial injection of KORD-AAV, GFP-AAV, or saline as a sham control (Ns per group: KORD=13, GFP=5, saline=6 for Cohort 1, and KORD=12, GFP=6, saline=6 for Cohort 2, plus an additional 2 mice to confirm viral expression at 3 weeks). Mice were given a recovery period of 3 weeks to allow for sufficient viral expression before undergoing training and testing in object recognition (OR) and object placement (OP) (Fig. 2). Each task was separated by a minimum of one week to ensure any effects of RE inactivation dissipated by the next inactivation. Mice in Cohort 1 were used to investigate pretraining inactivation of RE in OR and OP, while mice from Cohort 2 were used to investigate post-training RE inactivation. A minimum of one week after the completion of behavioral testing, mice from Cohort 1 were injected with SALB, trained, and perfused 1 hr later, and brains were

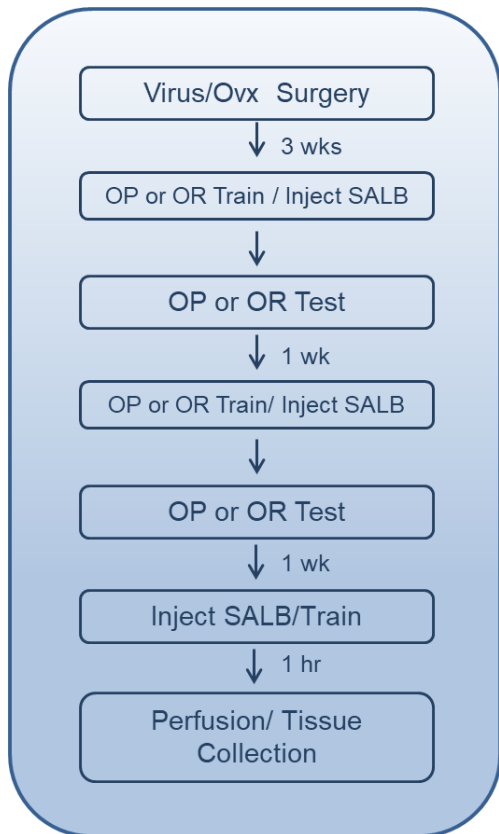


Figure 2. Flowchart depicting the timing of behavioral testing and tissue collection

Surgical Procedures

A minimum of 4 days after arrival in the vivarium, mice underwent a single surgical procedure for bilateral ovariectomy and intracranial administration of AAV (or control) to target the RE. Ovariectomy eliminates the primary source of circulating estrogens, which affect spatial memory in mice (Frick & Berger-Sweeney, 2001). Although this study did not investigate estrogenic effects in memory, mice were ovariectomized to be consistent with previous laboratory methods that were established using ovariectomized mice and allow for consistency in any follow-up studies that could investigate the combination of estrogenic effects and the role of RE in memory. The day prior to surgery, mice were moved from group housing to single housing. Mice were anesthetized with 5% isoflurane

collected to determine viral placement and for immunohistochemistry. Four mice (two from the KORD group, 1 GFP, and 1 saline) were assigned as homecage controls and did not receive any injection or training before perfusion. For all analyses, GFP and saline control groups were compared using a two-sample *t* test to ensure no significant differences between the means of both groups, then combined together into one Control group. Mice who had off-target viral placement or who did not explore the object for a sufficient time were excluded from analyses.

in 100% oxygen for induction, and placed in a stereotaxic apparatus, with anesthesia maintained at 2-2.5% isoflurane in 100% oxygen. Bilateral ovariectomy was conducted according to previously used methods (Lewis et al., 2008). Briefly, bilateral incisions were made through the skin and muscle wall in the upper dorsal pelvic region, and then the ovaries and tips of the uterine horn were ligated with Nylon suture material (Monomid) and removed. Uteri were returned to the body cavity, the muscle wall sutured, and skin incision closed with wound clips. Immediately after ovariectomy, AAV8-CAMKIIa-HA-KORD-IRES-mCitrine (Duke Vector Core, titer 4.2×10^{12} vg/mL), AAV8-CAMKIIa-eGFP (Duke Vector Core, diluted in sterile saline to 4.2×10^{12} vg/mL), or sterile saline (sham control) was infused into the RE resulting in 3 groups: 1) KORD, 2) GFP, and 3) Saline. 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 at a single point at a 15 degree angle to avoid hitting the midline sinus. Thus, the coordinates calculated for infusion were: AP: -0.6 mm; DV: -4.0 mm; ML: 0mm relative to Bregma. The needle was left in place for 2 minutes, and was then withdrawn 0.1 mm to create a pocket in which the virus could diffuse (Tuscher et al., 2018). Virus (or saline) was infused at a rate of 0.1 μ L/min for 5 min (0.5 μ L total), and the needle remained in place for 8 min after infusion to allow time for diffusion. The syringe tip was slowly withdrawn to prevent aspiration of liquid back into the syringe. After completion of virus infusion, the incision site was closed with chromic gut suture and antibacterial ointment (Neosporin) applied to the wound. Wound clips were removed after one week, and mice were given a minimum of three weeks before the onset of behavioral testing to allow for recovery and sufficient viral expression.

Drugs and Injections

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, gently warmed to 40 °C, and vortexed to ensure that the compound was fully dissolved. Drugs were injected subcutaneously at a dose of 10 mg/kg. Mice were weighed 1-2 days prior to behavioral testing to avoid additional stress on the testing day.

Object Recognition and Object Placement Testing

Episodic memory was tested using the object recognition (OR) and object placement (OP) tasks, which assess object recognition and spatial memory, respectively. These single-trial tasks are advantageous in that they rely on subject's innate preference for novelty, rather than appetitive or aversive motivating stimuli to influence behavior. Research from several laboratories has demonstrated that both the DH (Assini et al., 2009; Baker & Kim, 2002; Barrett et al., 2011; Cohen et al., 2013) and mPFC (Akirav & Maroun, 2006; Tanimizu et al., 2018; Tuscher et al., 2018) are essential for OR and OP memory consolidation.

Training and testing were conducted in a white open field box (width, 60 cm; length, 60 cm; height, 47 cm). To habituate mice to experimenter handling, they were handled for 1 min/day for 3 days. For two subsequent days, mice were then habituated to the empty open field box and allowed to roam freely for 5 min. During the training phase of both tasks, mice were exposed to two identical objects in the northeast and northwest corners of the box and allowed to accumulate 30 sec exploring the objects. Object

exploration was defined as direct contact between the nose and the object. Immediately after 30 sec of exploration was accumulated, mice were removed from the box, injected with SALB to target the memory consolidation process, and returned to their home cages. For pretraining injections, mice received SALB 10 minutes prior to training instead of immediately after training.

Memory was tested 24 or 4 hrs later, respectively, time points at which vehicle-treated mice remember the identity and location of training objects (Fortress et al., 2013). During OR testing, one training object was replaced with a novel object. During OP 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. Mice were given 20 min to accumulate 30 sec of exploration. 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 a different object. Any mouse who did not explore for at least 27 sec on any training trial or 30 sec on a testing trial for a given task was excluded from analysis in that task. Testing in OR and OP was separated by at least one week to ensure the effects of SALB injection and subsequent inactivation had dissipated.

Time spent with the objects, time to accumulate 30 sec of exploration, and pathlength were recorded using AnyMaze software (Stoelting). Because mice prefer novelty, mice who spent significantly greater time with the novel object (OR) or moved object (OP) than chance (15 sec) were considered to have intact memory for the identity and location of the original training objects.

Tissue Collection and Slide Preparation

To study the effects of RE inactivation on neural activity in the DH, mPFC, and RE during consolidation, mice were trained with objects as described above and activity was then measured by quantifying levels of the immediate early gene EGR-1. To determine the effects of RE inactivation on training-induced EGR-1 activity in the DH, as well as determine the extent to which KORD-mediated inactivation affected EGR-1 expression in the RE, mice were trained with two objects and were perfused 1 hr later, a timepoint at which EGR-1 has previously been shown to be elevated after behavioral training (Huckleberry et al., 2015; Lonergan et al., 2010). Mice who did not accumulate at least 15 sec of exploration within the 20-min session were excluded from the study. Four mice were not trained before perfusion to serve as homecage controls.

A minimum of one week after completion of behavioral testing, mice from Experiment 1 were exposed to two identical novel objects or left in the homecage as the controls described above (Homecage group, $N=4$). Mice in the novel-object group were injected with SALB 10 min prior to the start of training, and were then given up to 20 min to accumulate 30 sec of exploration. Immediately after completion of 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 0.1M 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 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 $-20\text{ }^{\circ}\text{C}$, and mounted

onto Superfrost Plus slides (Fisher Scientific) or stored at -20 °C in a cryoprotectant solution until immunohistochemistry. Two mice were excluded from analysis: one in the KORD group with off-target viral expression, and two (one KORD, one Control) that did not accumulate at least 15 s of object exploration.

To check viral placement, 1 in every 6 sections of the RE from eGFP-expressing mice was mounted to a slide, coverslipped with aqueous mounting medium containing DAPI (Santa Cruz), and imaged under a fluorescent microscope. For saline animals, 1 in every 6 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 made with the aid of a mouse brain atlas (Paxinos and Watson, 2001).

Immunohistochemistry

The KOR-DREADD is fused to an epitope tag (HA) that allows for more sensitive labeling than the fluorophore mCitrine provides. Tissue was washed 3x10 min in 25 mM PBS to remove cryoprotectant and treated with 1% Sodium Borohydride (NaBH₄) for 15 min, and then rinsed 2x5 mins in PBS, blocked in 10% Normal Goat Serum (NGS, Biogenex), and incubated overnight at 4 °C in HA-tag rabbit primary antibody (1:1000, Cell Signaling #3724). The next day, tissue was washed 3x5 min, incubated in Alexa-Fluor 594 goat anti-rabbit IgG secondary (1:500, ThermoFisher #A-11012) for 90 min at room temperature, washed 3x5 min, mounted onto glass slides, and coverslipped with an aqueous mounting medium containing DAPI (Santa Cruz). Tissue was protected from light at all times to avoid photobleaching of the mCitrine tag.

EGR-1 labeling

Immediate Early Genes (IEGs) are rapidly activated in response to stimuli or neuronal activation. EGR-1 is a zinc-finger transcription factor (also known as Zif268). While always present at basal levels in the nuclei of cells, expression of EGR-1 is upregulated in neurons in response to neuronal activity (Duclot & Kabbaj, 2017). EGR-1 mRNA and subsequent protein expression increases after exposure to episodic and spatial memory tasks (Guzowski et al., 2001; Lonergan et al., 2010; Poplawski et al., 2014). Furthermore, knockout of EGR-1 impairs recognition memory (Bozon et al., 2003), whereas overexpression enhances synaptic plasticity and recognition memory consolidation (Penke et al., 2013). Therefore, measuring EGR-1 expression in neurons can be an indirect way to measure the neuronal activity that accompanies memory observed in behavioral tasks.

Tissue was washed in 25 mM PBS to remove cryoprotectant, blocked with 5% NGS in 25mM PBS with 0.3% TritonX-100, and incubated overnight at 4C 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.

Image Acquisition and Analysis

Z-stack images (15 slices per stack, step size 1.22 μm) were acquired on a confocal microscope at 20x magnification (Olympus FV1200), 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 et al., 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 blind to treatment condition.

Statistical Analyses

Prism 8 (GraphPad) was used for all statistical analyses. To assess learning within each group, OR and OP data were analyzed using two-way 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*-test), 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 was also analyzed using two-tailed *t* tests. IHC data were analyzed using one-way ANOVA, and, where appropriate, Tukey posthoc tests were conducted to assess differences among the homecage, training control, and training KORD groups. Significance was set at $p < 0.05$ for all tests.

RESULTS

Expression of Viral Constructs in the RE

To confirm expression of viral constructs after 3 weeks as previously reported (Tuscher et al., 2018), two mice underwent infusion of AAV (one GFP and one KORD) and were perfused after 3 weeks. Viral expression was made visible by expression of fluorescent proteins eGFP in the Control AAV and mCitrine in the KORD-AAV. Both the

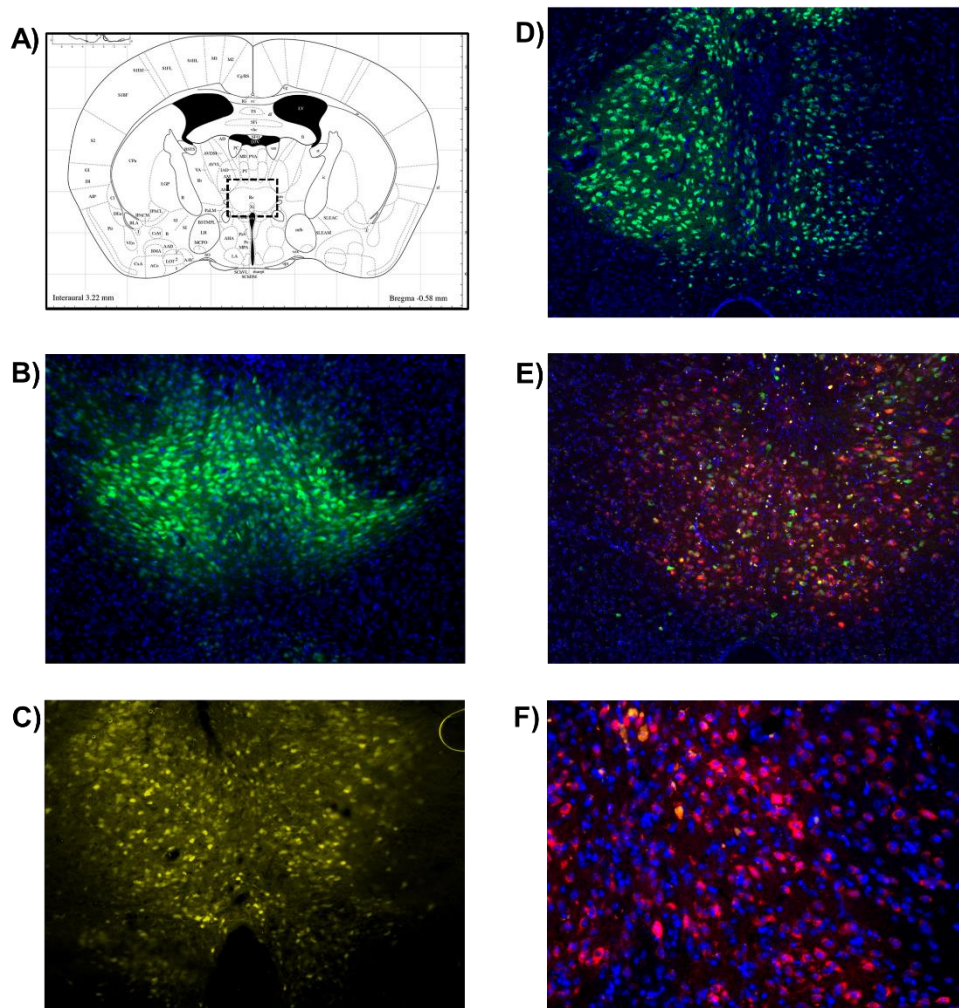


Figure 3. Representative Viral Expression in RE. (A) Target coordinates according Paxinos and Watson brain atlas (2001). Dotted black box indicates region imaged in (B-E). (B) Expression of eGFP in RE (green) counterstained with DAPI (blue) as well as mCitrine (yellow) in KORD mice (C) was present at 3 weeks after surgery (10 x magnification). (D) Representative expression of eGFP present after completion of behavioral testing (10 x magnification) (E) Representative expression of mCitrine (green), expression of KORD-HA epitope tag (red) and overlap between the two (yellow) at 10x magnification after completion of behavioral testing. (F) mCitrine and HA tag expression at 20x magnification after completion of behavioral testing.

GFP-AAV and KORD-AAV constructs were readily visible three weeks after infusion (Fig. 3B,C) and several weeks later after completion of behavioral tasks (Fig. 3D-F). eGFP could be seen throughout the RE on both sides of the midline (Fig. 3B, D). In mice infused with the KORD, mCitrine (yellow in single-color image Fig. 3C, green in multichannel images Fig. 3E,F) and the HA epitope tag (red) could be seen throughout the RE, with areas of colocalization indicated in yellow (Fig. 3E, F). These representative images suggest robust expression of the GFP and KORD viruses three weeks after infusion that persisted throughout the duration of the experiment.

Inactivation of the RE Before or Immediately After Training Impairs Spatial Memory

Pre-training inactivation: To determine whether RE is necessary for OR and OR memory acquisition, ovariectomized mice were infused with saline, GFP-AAV, or KORD-AAV at least three weeks prior to training to allow sufficient time for optimal virus expression. In Cohort 1, 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. 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. 4A), whereas the KORD group did not ($t_{(10)} = 0.7269$, $p = 0.484$; Fig. 4A). Moreover, the KORD group spent significantly less time with the moved object than the Control group, as determined by unpaired, two-tailed t test ($t_{(19)} = 2.309$, $p = 0.0323$; Fig. 4A). Combined, these data suggest 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 30s 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. 4B) or the time to accumulate 30 sec exploration ($t_{(19)} = 0.9134$, $p = 0.3725$; Fig. 4C), indicating that RE inactivation did not affect exploratory motivation or ability.

Post-training inactivation: In Cohort 2, mice 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. Similar to Cohort 1, Control mice tested in OP spent significantly more time than chance with the moved object ($t_{(8)} = 4.413$, $p = 0.0022$; Fig. 4D), whereas the KORD group did not ($t_{(8)} = 0.6067$, $p = 0.5609$; Fig. 4D), demonstrating impaired spatial memory consolidation. An unpaired t test suggested a trend towards a significant impairment in the KORD group relative to Controls ($t_{(16)} = 1.945$, $p = 0.0696$). As in Cohort 1, there were no significant differences in path length ($t_{(16)} = 1.172$, $p = 0.2583$, Fig. 4E) or time to accumulate 30 sec exploration ($t_{(16)} = 1.782$, $p = 0.0937$, Fig. 4F).

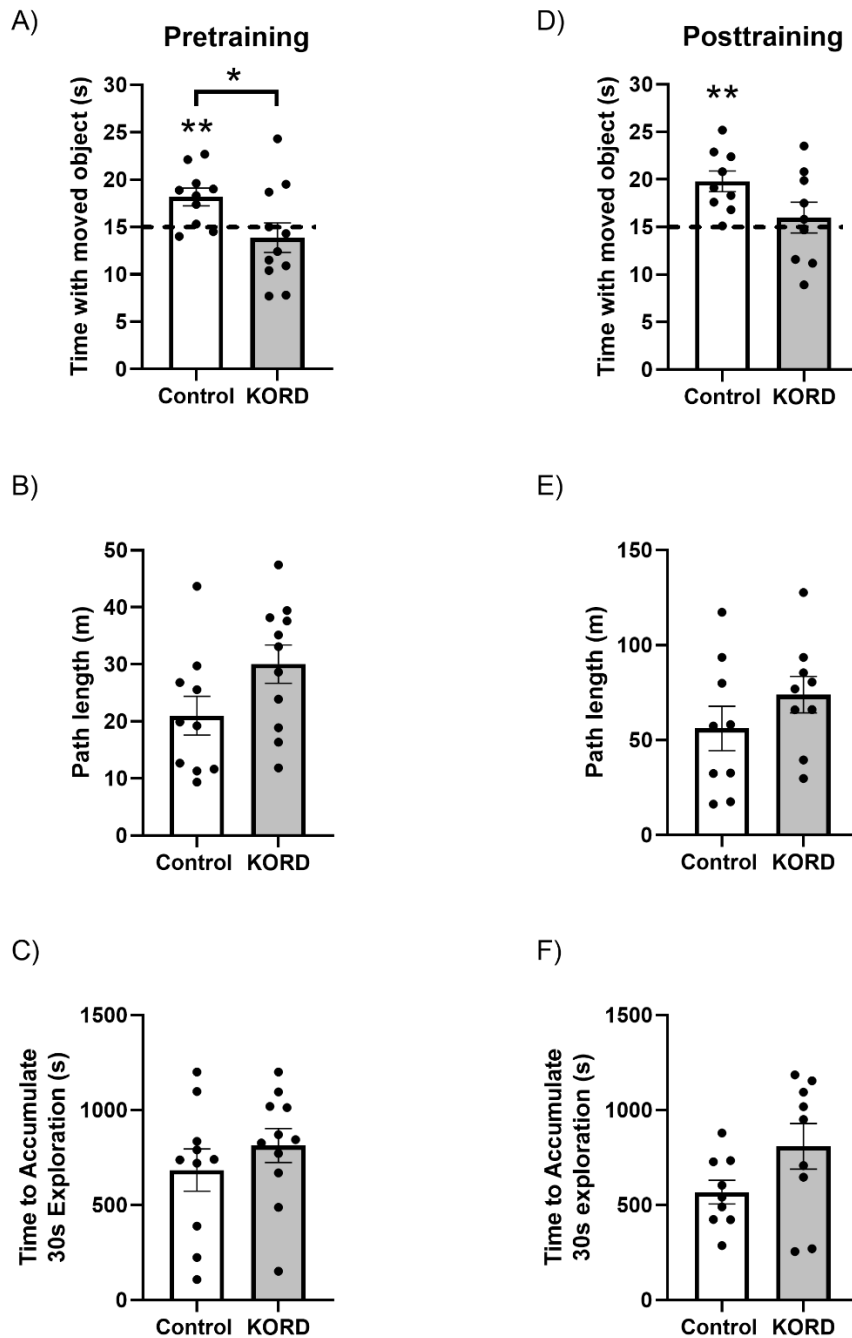
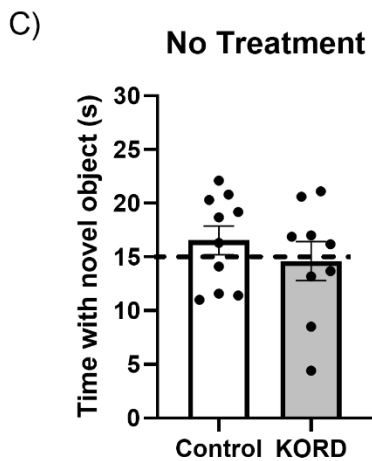
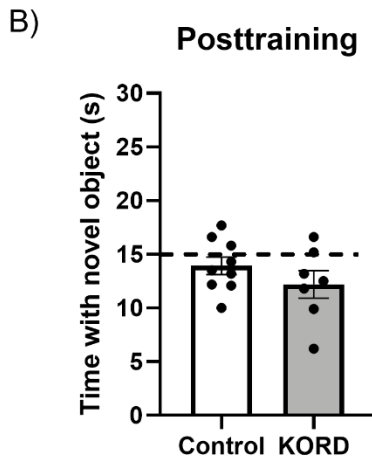
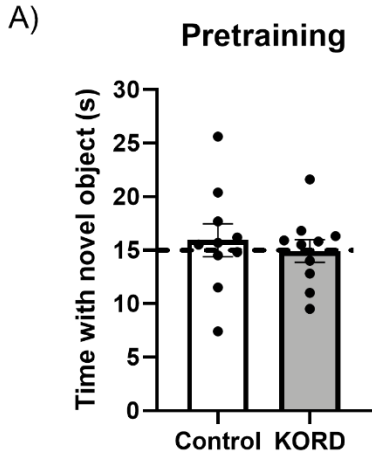


Figure 4. Inactivation of the RE Before or Immediately After Training Impairs OP Memory. (A) In Cohort 1, control mice, but not mice expressing the KORD, spent more time than chance (dashed line at 15 sec) with the moved object (** $p < 0.01$). The control group also spent significantly more time with the moved object than the KORD group (* $p < 0.05$). Control and KORD mice did not significantly differ in exploration path length (B) or the time to accumulate 30 s exploration (C) 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. (D) Control mice in Cohort 2 injected with SALB immediately after training spent significantly more time than chance with the moved object (** $p < 0.01$), whereas KORD mice did not, indicating impaired memory. Both groups of mice did not significantly differ in exploration path length (E) or time to accumulate 30s exploration (F) ($p > 0.05$ for both measures). $n = 9-11$ per group.

Indeterminate Effect of RE Inactivation on Object Recognition Memory



In both cohorts, OR memory was tested using the same procedures as OP, with the exception that one object was replaced with a novel object, and the delay was increased to 24 hours in accordance with previously published research from our laboratory (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Zhao et al., 2010).

Unexpectedly, Control mice in both cohorts failed to show evidence of learning, precluding observations about the effects of RE inactivation on object recognition memory acquisition or consolidation. Among mice receiving pretraining injection of SALB, neither the Control group ($t_{(9)} = 0.605$, $p = 0.5601$; Fig. 5A) nor the KORD group ($t_{(9)}$

Figure 5. Indeterminate Effect of RE Inactivation on OR Memory.

In OR testing in both cohorts, neither the Control nor KORD group spent significantly more time than chance with the novel object when SALB injections were given pretraining (A) or post-training (B). (C) To examine potential effects of injection stress, mice in Cohort 2 were tested again in OR but without a post-training injection of SALB. However, neither group spent significantly more time than chance with the novel object. Because the Control group did not display intact memory, a conclusion about the effect of RE inactivation on object recognition memory cannot be made. However, the stress and effect of SALB injection does not appear to be responsible for the lack of memory. $n = 7-10$ per group.

= 0.07507, $p = 0.9418$; Fig. 5A) spent significantly more time than chance with the novel object. Likewise among mice receiving post-training injection of SALB, neither the control group ($t_{(8)} = 1.314$, $p = 0.2254$; Fig. 5B) nor the KORD group ($t_{(6)} = 2.155$, $p = 0.0746$; Fig. 5B) spent significantly more time than chance with the novel object.

Due to our repeated and unexpected findings that control mice did not display intact memory for the training objects, we tested the Cohort 2 mice again without a post-training injection of SALB to eliminate a potential source of stress that could interfere with memory. However, neither group spent significantly more time with the novel object during training (control: $t_{(9)} = 1.154$, $p = 0.2784$, KORD: $t_{(8)} = 0.2087$, $p = 0.8399$; Fig. 5C), suggesting that injection stress was not likely responsible for the unexpected memory impairments. At present, it remains unclear why control mice did not show evidence of learning using a delay between training and testing at which controls typically exhibit intact memory (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Koss & Frick, 2019; Zhao et al., 2010). Unfortunately, this lack of learning in the control group means that a conclusion about the effects of RE inactivation on object recognition memory cannot be made at this time.

Effects of RE inactivation on EGR-1 expression after object training

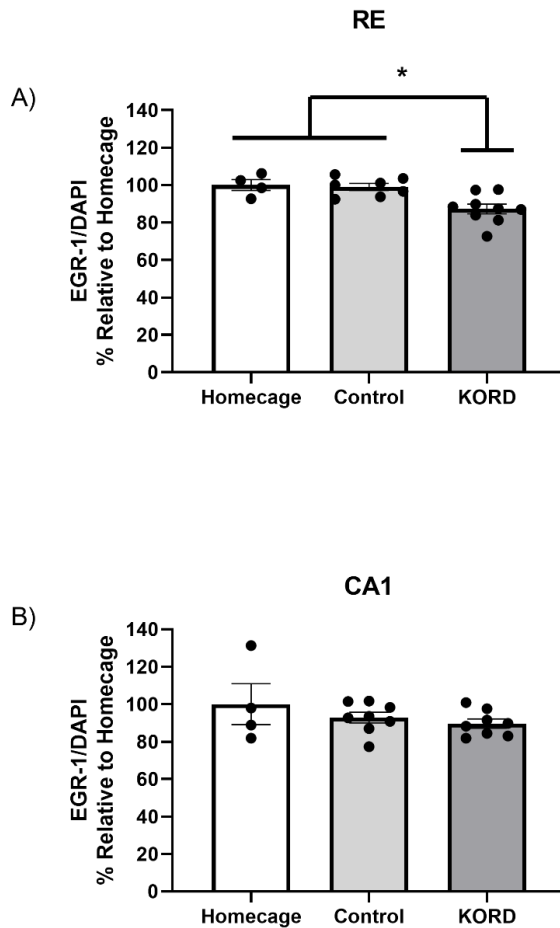


Figure 6. Effects of RE inactivation on EGR-1 expression

(A) RE inactivation via KORD decreased EGR-1 expression in the RE compared to the homecage and control groups (* $p < 0.01$). (B) In CA1, the groups did not differ in EGR-1 expression ($p > 0.05$). These data suggest that KORD-mediated inactivation of excitatory neurons reduced EGR-1 expression in the RE but not CA1. $n = 4$ (homecage), 7 (control), 9 (KORD).

To investigate whether RE inactivation reduces training-induced elevations in neuronal activity, mice from Cohort 1 were injected with 10 mg/kg SALB 10 min prior to training and then explored to two novel objects for 30 sec. They were then perfused 1 hr later. Four mice were left in their homecage as controls and were perfused after the trained mice. Protein levels of the immediate early gene EGR-1 were then measured via fluorescent immunohistochemistry in RE and the CA1 pyramidal layer in the DH.

In the RE, the main effect of treatment was significant ($F_{(2,17)} = 8.512$, $p = 0.0027$, Fig 6A). A Tukey multiple comparisons test revealed

that EGR-1 expression in the KORD group was significantly lower compared to both homecage ($p = 0.0127$) and Control ($p = 0.0064$) groups (Fig 6A). In the DH, the main effect of group was not significant ($F_{(2,17)} = 1.091$, $p = 0.3582$, Fig 6B).

DISCUSSION

The primary goal of this thesis was to determine the necessity of the RE in OR and OP memory. On the basis of previous evidence suggesting a role for the RE in mediating hippocampal-dependent memories, we hypothesized that RE inactivation would block memory acquisition and consolidation in two hippocampal-dependent tasks. 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 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 in the RE, but not the CA1, confirming reduction of neural activity in the RE by the KORD manipulation. Although we also attempted to examine effects of RE inactivation on the acquisition and consolidation of object recognition, the failure of control mice to learn in this task prevented assessment of RE inactivation. Taken together, these data suggest that RE plays a role in spatial memory, particularly during acquisition and the early consolidation window.

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 and 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 and female 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. Because both mouse studies found an impairment after RE inactivation, it is possible that RE is essential for OP memory in mice, but not rats. Differences in inactivation technique and OP testing protocols could also play a role. Nevertheless, the present data suggest a potentially important contribution of the RE to OP memory formation that warrants further investigation.

It should also be noted that our OP findings are consistent with literature using reversible RE inactivation reporting a role for the RE in other hippocampal-dependent tasks. 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 et al., 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 hippocampal-dependent memory.

However, this conclusion is complicated by inconsistencies among studies examining a role for the RE in other spatial memory tasks. 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 et al., 2009). However, ibotenic acid lesions of RE in rats influenced swimming strategy on a MWM probe trial, but did not affect overall learning of the platform location during acquisition (Dolleman-van der Weel et al., 2009). Likewise, NMDA-induced lesions of RE in rats did not impair acquisition or 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, another study found that muscimol-induced RE inactivation in rats during the testing phase of a crossword-like maze impaired cue-based spatial navigation ability, whereas inactivation after each daily training (targeting the consolidation phase) had no effect on memory (Mei et al., 2018). These discrepant findings could result from several methodological factors, including inactivation method and differences in task protocols, task complexity, duration of training, and length between training and probe testing. Taken together, the results of these studies suggest that the method of inactivation (lesioning vs. pharmacological inactivation) and task parameters are important factors to consider when investigating the RE's role in spatial memory.

Although we had hoped to establish a more general role of the RE in mediating multiple forms of hippocampal-dependent memory by examining both OP and OR memory, we were not able to find evidence of a role for the RE in OR memory because controls did not display preference for the novel object at 24 hours after training. This surprising outcome is inconsistent with many previous findings from our laboratory showing that vehicle-infused mice of both sexes display intact OR memory consolidation

using this delay (Boulware et al., 2013; Fernandez et al., 2008; Fortress et al., 2013; Koss & Frick, 2019; Zhao et al., 2010). To determine if the stress of SALB injection could have influenced OR memory, we repeated the experiment without SALB injection; however, controls in this replication also did not show evidence of memory. Because both sham and GFP controls exhibited memory impairments, it is possible that the intracranial surgical procedure to target RE may have caused small but sufficient damage such that OR memory was impaired at 24 hours. However, the small size of the infusion syringe and short duration of the infusion procedure makes this unlikely. Other factors could have contributed, including the type of objects used and experimenter identity. However, we find these possibilities unlikely because OR was repeated at least once in both cohorts with different object pairs that had previously yielded learning in other experiments. Furthermore, a small set of trials were re-scored by another experimenter and found to agree with the original scoring. Ultimately, the reasons for these unexpected results are unclear based on the current data. Additional testing with new mice will have to be conducted to determine a potential role for RE activity in OR memory.

To investigate whether DREADD-mediated inactivation of the RE affected learning-induced activity in the RE and pyramidal layer of CA1, control and KORD mice received an injection of SALB prior to object training and were perfused 1 hr later for immunohistochemical labeling of EGR-1, an immediate early gene that has been used as an indirect measure of neuronal activity. As a control, two mice from each group were left in their homecage before perfusion and did not receive SALB or object training. In the RE, training did not significantly increase EGR-1 expression in control mice relative to homecage; however, RE inactivation in the KORD mice significantly reduced EGR-1

expression relative to both homecage and control mice. Although no other studies have examined EGR-1 expression in the RE, a few studies have measured RE expression of the immediate early gene cFos after training in other tasks. One study found that cFos expression increased in the RE after a MWM probe trial given 25 days after training but not 5 days after training, suggesting that the RE is most active when retrieving a remote memory (Loureiro et al., 2012). The number of Fos-positive cells also increased after acquisition, but not retrieval, of trace fear memory (Lin et al., 2020). Additionally, Fos-positive cells were increased in male rats exposed to a novel environment (Kinnavane et al., 2017). Although the objects were novel for each training trial in this study, mice were well adjusted to the testing environment by the end of the study and thus the relative novelty of the final object training trial may be reduced compared to that of the environments used in the aforementioned studies of cFos expression in RE.

Likewise, we did not observe an increase in EGR-1 levels in the pyramidal layer of DH CA1 after object training. Training-induced EGR-1 expression may peak at a time point other than 1 h after training. Alternatively, object training may not be sufficient to increase EGR-1 expression, compared to other conditions such as contextual fear conditioning, novel environment exposure, and MWM training, in which increases in EGR-1 have been observed (Huckleberry et al., 2015; Lonergan et al., 2010; Pollak et al., 2005). One study found that neither OR training nor retrieval (trial duration 15 min and 10 min, respectively) increased EGR-1 mRNA in whole hippocampus at either 30 min or 2 hr post-training, although there was a robust increase in BDNF mRNA at 30 min (Romero-Granados et al., 2010). Importantly, as the RE synapses onto both excitatory pyramidal neurons and inhibitory interneurons in the CA1 (Dolleman-Van der Weel et al., 1997;

Dolleman-Van der Weel & Witter, 2000), it is possible that inactivating RE interrupts the pattern of activity in the pyramidal layer of CA1 necessary for spatial memory formation without changing the overall level of activity in the region. Future studies may investigate cFos expression or other cellular or molecular markers associated with learning such as dendritic spine density, which we have found to increase in the CA1 of ovariectomized mice within 40 min of training (Kim et al., 2019).

In conclusion, the present study finds evidence that temporary inactivation of the RE using an inhibitory KOR-DREADD impairs spatial memory acquisition and consolidation in ovariectomized mice. This study is one of very few that have investigated the RE's role in memory in mice, and the first study of which we are aware that used female subjects. As such, these data add to a growing body of literature defining the RE's role in memory. Better understanding the RE's contributions to memory formation will provide important new insights into the complex network of brain regions facilitating episodic and episodic-like memory.

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