Brief Research Report

The thalamic reuniens is associated with consolidation of non-spatial memory too.

Hamilton, J. J.*[1,3], Dalrymple-Alford, J. C. *[1,2,3]

1. School of Psychology, Speech and Hearing, University of Canterbury, Christchurch, New Zealand
2. New Zealand Brain Research Institute, Christchurch, New Zealand
3. Brain Research New Zealand – Rangahau Roro Aotearoa

* Correspondence: Jennifer J Hamilton or John C Dalrymple-Alford.
Email addresses: jenny.hamilton@canterbury.ac.nz or john.dalrymple-alford@canterbury.ac.nz

Running title:
Thalamic reuniens and non-spatial memory

3 figures
Manuscript word count (including title): 3474
Abstract word count: 185

Keywords: nucleus reuniens, memory, consolidation, recall, paired-associate, non-spatial

Abstract
The nucleus reuniens (RE) in the ventral midline thalamus may provide a critical node between the hippocampus and the prefrontal cortex (PFC) to facilitate systems memory consolidation. Thus far, evidence suggests that the RE has a role in the consolidation of spatial memory and contextual fear memory, but not for non-spatial memory. Here, we trained intact adult male rats to learn an arbitrary odor-object paired-association and then examined neural activation in brain structures (using Zif268) after a post-acquisition test. Neurons in the RE showed markedly increased activation when rats were re-tested for the previous non-spatial memory 25 days after acquisition training, relative to a group tested at 5-days post-training, as well as a control group tested 25 days after acquisition but with a new pair of non-spatial stimuli, and home cage controls. The remote recall group also showed relatively augmented Zif268 expression in the superficial layers of the medial PFC (anterior cingulate and prelimbic cortices). These findings support the conclusion that the RE is preferentially engaged during remote recall in this non-spatial task and thus has a role beyond spatial memory and contextual fear memory.

1. Introduction

The nucleus reuniens of the thalamus (RE) is ideally situated to mediate information exchange between hippocampus and prefrontal cortex (PFC) and participate in a variety of higher cognitive functions (reviews: Dolleman-van der Weel et al. (2019); Ferraris et al. (2021); Mathiasen et al. (2020). One of the most interesting lines of research suggests that the RE, and the closely situated rhomboid nucleus (RH), are critical for long-term memory consolidation. Based on lesion and inactivation studies in rats, these thalamic nuclei influence accurate recall 25 days after acquisition, but not short-term retrieval after 5 days, for both spatial memory (Klein et al., 2019; Loureiro et al., 2012) and contextual fear memory (Quet, Majchrzak, et al., 2020).

Neuronal activity based on immediate early gene (IEG) expression using the c-Fos marker also supports the involvement of RE in remote retrieval of spatial memory in the water maze (Klein et al., 2019; Loureiro et al., 2012). Another study reported that the long-term retrieval of remote contextual fear memory also engaged the RE, PFC and amygdala (Silva et al., 2019), but this is not always evident (Quet, Majchrzak, et al. (2020). Together with lesion evidence, these findings suggest that the RE facilitates a dialogue between the PFC and the hippocampal system to support long-term memory consolidation and dynamic changes from a primarily hippocampal-dependent process to one that increasingly engages PFC activity. Despite a role for the RE in remote memory recall when spatial information is required or when context refers to the background of diffuse multimodal stimuli, the broader relevance of the RE for memory consolidation may be limited. Specifically, a recent lesion study reported that the RE was not critical for long-term nonspatial, social-olfactory memory (Quet, Cassel, et al., 2020). These authors suggested that the social transmission of food memory is not hippocampus-dependent to explain why the RE is not involved in consolidation in this non-spatial task.

The last point connects with debate as to whether the hippocampal system is responsible primarily for the acquisition of spatial and context memory or more general cognitive and relational representations. One critical issue is the presence of an explicit temporal feature of the memory (Ranganath, 2019; Whittington et al., 2022). The question remains, then, whether the engagement of the RE for long-term consolidation is limited to spatial and context memory specifically or more general processes that are sensitive to hippocampal dysfunction. In rats, the acquisition of a paired-association between arbitrary object and odor stimuli is dependent on the hippocampus only when a 10-second temporal lag is introduced between the to-be-associated stimuli (Gilbert & Kesner, 2002; Kesner et al., 2005). Here, we provide evidence that the RE shows increased IEG activation during remote, relative to recent, recall of a previously acquired non-spatial association that incorporates such a temporal lag.

2. Materials and methods

2.1. Animals and housing conditions

Male Long-Evans rats, aged 12 months, were group-housed in Makrolon cages (48x28x22cm) on a reversed 12-hour light-dark cycle. Rats were maintained at 85% *ad libitum* body weight with testing during the dark phase. All procedures were approved (University of Canterbury Animal Ethics Committee and complied with ARRIVE guidelines). Three groups were established by random assignment. Post-acquisition, one group was tested for recent retention (5-days post-acquisition; n=8), the second for remote retention (25-days post-acquisition; n=8), and the third on a new association at 25-days post-acquisition (25-day New, n=6). There was also a home cage control group (n=9).

2.2. Non-spatial paired-associate memory task

For details on the apparatus and procedures, see Hamilton and Dalrymple-Alford (2022). Training on simple odor and simple object discriminations was conducted first to familiarise rats with the general "go/no-go" procedures. Acquisition for an odor-object paired-association was conducted in the same red Perspex runway (93x26x26cm high) with vertically removable doors (26x26cm; Figure 1). The odor (e.g. 20ul of lemon or clove; Essential Oils, New Zealand) was mixed with 5ml of sunflower oil, and placed on a sponge (6.5x6x8mm) surrounding a plastic cap at the centre of the door at the end of the second compartment (15cm); the cap presented a 1mg piece of chocolate, available after the rat was released from the 15cm-long start compartment. After removal of the door with the odorised sponge, the rat was retained for 10 seconds in a third compartment (15cm). It was then allowed to run to the end of the runway to interact with a lightweight and visually distinct object. The object's base was hinged to a food well beneath so the rat could search for additional chocolate reward. The presence of the second reward depended on whether the specific odor-object pairing was a "correct" association. Black curtains enclosed the test area to minimise spatial cues. The observer sat within the curtains, adjacent to the runway and operated the doors.

Each rat received 12 massed daily trials, six go (rewarded) and six no-go (non-rewarded) in a pseudo-randomized order, with no more than three of either type run consecutively within session. Correct pairings for the paired-associate task were counterbalanced across rats. The critical measure was latency to push (nose or paw) the object after opening the last door. Rats learned that two odor-object pairings were rewarded under the object (e.g. Odour 1 + Object A and Odour 2 + Object B) and that the two alternate pairings were not rewarded (i.e. Odour 1 + Object B and Odour 2 + Object A). A trial was 'correct' if the rat responded within 8 seconds on go trials or withheld responding for longer than 8s on no-go trials. Rats that reached a criterion of 80% correct trials over 3 consecutive days, after a minimum of 25 sessions (N = 15), were removed from further training and re-tested at either 5 days or 25 days later. There were 8 rats that continued for the maximum of 50 days of training, but all of these showed clear evidence of acquisition (3 rats each in Recent and Remote recall groups and 2 in the 25-day New group); their final latency difference between go and no-go trials was > 3.3 seconds with a mean of 3.8 seconds).

2.5. Post-acquisition retention test

Retention was conducted 5 days (recent recall) or 25 days (remote recall) after reaching criterion or else 50 days of training. The retention session used the same previously-learned association for the Recent and Remote groups. For the 25-day New learning group, a completely new odor-object pairing was used per rat; this assessed the impact of a delayed return to testing conditions without explicit recall of the previously acquired association.

2.6. Histology

Immediately following the post-acquisition test (Recent, Remote, and 25-day New), or straight from an individual holding cage (Home Cage controls), rats were placed in a familiar dark, quiet room for 90 min prior to perfusion. Rats had experienced this procedure on two previous days. The rat was deeply anaesthetised with sodium pentobarbital (125 mg/ kg) and perfused transcardially with saline

followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Brains were post-fixed in 4% PFA and at least 48 h later placed in a long-term solution (20% glycerol in 0.1 M PB). Coronal 40-µm sections were collected using a freezing microtome (Thermo Fisher, UK) and stored in a cryo-protectant solution (30% glycerol, 30% ethylene glycol in 0.1 M PB) at -20°C prior to immunohistochemistry.

Free-floating sections were incubated in rabbit polyclonal Zif268 primary antibody (Egr-1; 1:1000 Cat# sc-110; Santa Cruz Biotechnology, USA) for 72 h at 4°C in PBSTx with 1% NGS, then incubated in biotinylated goat antirabbit secondary antibody (1:1000: Vector Laboratories BA-1000) overnight in PBSTx and 1% NGS. Following DAB visualisation (16-20 min, pending visual check) and mounting, Zif268-positive cell staining in each region of interest was photographed with a 10x objective using a light microscope (Leica, Germany). Automated cell counts were obtained through ImageJ analysis software (National Institute of Health, NIH, USA). The regions of interest were manually selected and images converted to 8-bit grey scale, background was subtracted (rolling = 40), converted to mask and the watershed function applied. All neuronal cells above threshold ('MaxEntropy' threshold, circularity 0.65–1.0) were counted. Between two and six sections per region of interest were quantified per rat to obtain the average Zif268-positive cell count per mm^2. Sections through the intralaminar thalamic nuclei (ILt) were counted manually due to technical issues on the microscope.

Regions of interest were selected for their potential involvement in memory recall. This included: the medial PFC (A32D, anterior cingulate region; A32V, prelimbic region); hippocampus (dorsal and ventral CA1 and CA3); midline thalamic RE and RH; centromedial, and paracentral ILt; and the retrosplenial cortex (dysgranular Rdg, granular Rga and granular Rgb).

2.7. Data analysis

ANOVA using Statistica (v13; Dell Inc.) evaluated mean differences across the groups (Recent, Remote, 25-day New, and Home Cage). Repeated measures factors were added for blocks of trials for the paired-associate memory task and Zif268 counts across related regions or subregions of interest. A reciprocal transformation of latency data for individual trials was used to establish homogeneity of variance. On each test day, the transformed latencies for individual trials generated a mean latency for the six rewarded trials and six non-rewarded trials and the difference used to evaluate performance. Latencies were carried forward for acquisition for rats that reached criterion. The final forty acquisition days were analysed, as running was more consistent after the first 10 days. To account for multiple comparisons and balance Type I and Type II errors, we used the significance level of $p<0.02$ for behavioral analyses and $P<0.01$ across regions of interest for Zif268 expression. Post hoc Newman–Keuls (N-K) tests assessed pairwise group differences. Simple main effects analysis was used when there were significant interactions involving repeated measures factors. Insufficient or missing brain sections in some rats (N = 2) reduced the degrees of freedom for some analyses; removal of these cases from other analyses did not change the findings.

3. Results

3.1. Behaviour

Rats in all groups rapidly acquired both the simple odor and simple object discrimination tasks at a similar rate (data not shown; simple odor discrimination, Group main effect, $F(2,19)=0.08$, $p=0.92$; simple object discrimination, $F(2,19)=2.11$, $p=0.14$).

The mean latency difference (based on reciprocal latencies) for paired-associate acquisition and performance on the retention test (Recent, Remote) and on the new odor-object association (25-day New) is shown in Figure 1. An increasing latency difference was evident over training for the non-rewarded compared to the rewarded trials. All three groups acquired the odor-trace-object paired associate task in a similar fashion (Figure 1; Group main effect, $F(2,19)=0.52$, $p=0.60$; Block main effect, $F(7,14)=247.84$, $p<0.001$; Group x Block interaction $F(14,133)=0.71$, $p=0.75$). The three groups did not differ on the final block of acquisition (Group at Block 10, $F(2,19)=0.26$, $p=0.77$).

There was no correlation between mean latency difference in the final block of acquisition and the number of days to criterion or end of training ($r(21)=-0.14$, $p>0.5$).

Rats in the Recent and Remote recall groups showed clear retention of the acquired association, compared to rats in the 25-day New odor-object pairing ($F(2,19)=9.63$ $p<0.001$). Although the two recall groups exhibited similar responding on their last Block of acquisition (Block 10), the Recent recall group showed better retention than was shown by the Remote recall group ($p<0.02$).

3.2 Zif268

Figure 2 shows Zif268 expression in the RE, RH, and the medial prefrontal A32D (ACC) and A32V (prelimbic) regions of interest for the four groups (Recent, Remote, 25-day New, and Home Cage controls). In the RE, all three trained groups showed higher expression than was found in the Home Cage group (Group main effect $F(3,25)=64.72$, $p<0.001$), suggesting that engagement in and general memory for the behavioral task *per se* associates with increased activity in the RE. Critically, N-K post hoc analysis confirmed that all four groups differed from each other for Zif268 expression in the RE ($p<0.004$). Remote recall of the odor-object paired-associate memory produced markedly higher Zif268 expression in the RE compared to home cage group (by a factor of 298%; effect size $d = 9.91$), recent recall (161% increase; $d = 4.13$) and exposure to a new association at 25-days (120% increase; $d = 1.87$). To a lesser extent, both recent recall and exposure to the new association also produced greater levels of expression compared to Home Cage (Recent: 185% increase; $d = 4.25$; 25-day New: 247% increase; $d = 7.35$). There was no correlation ($r(19)=0.21$, $p>0.3$) between the mean latency difference score on the retention test and zif268 expression in the RE within the individual groups: Remote, $r(6)=0.24$, $p>0.6$; Recent, $r(7)=-0.60$, $p>0.1$; 25-day New, $r(5)=-0.11$, $p>0.8$). The days taken to reach criterion was also not associated with Zif268 expression in the RE ($r(19)=-0.31$, $p>0.17$).

A similar, albeit weaker, pattern of Zif268 expression to that of the RE was apparent in the RH (Group main effect, $F(3,25)=4.42$, $p=0.01$). However, post-hoc N-K revealed a significant difference only between Remote and Home Cage groups ($p=0.01$) with Recent and 25-day New producing non-significantly lower levels of expression compared to the Remote group ($p<0.10$).

There was no Group main effect for the A32D (ACC), aggregated across all three layers ($F(3,27)=1.18$, $p=0.35$). A Group x Layer interaction ($F(6,54)=3.49$, $p=0.005$) was evident with the Remote recall group showing higher Zif268 expression in the superficial Layer II than in both the Recent recall group ($F(1,27)=5.62$, $p=0.02$) and 25-day New learning group ($F(1,27)=6.16$, $p=0.01$). Zif268 expression in Home Cage group was not significantly different to any other group ($p<0.12$). There were no group differences for the deeper layers.

Across the cell layers of the A32V (prelimbic) region of the medial PFC, Zif268 expression showed a similar pattern to that seen in the ACC. That is, a Group x Layer interaction ($F9,81)=7.13$, $p<0.001$) was again driven by elevated expression in superficial Layer II in the Remote recall group, with expression in this group significantly higher compared to both Recent ($F(1,27)=7.66$, $p=0.01$) and 25-day New groups ($F(1,27)=13.56$, $p<.002$). The Home Cage group showed an intermediate level, but significantly higher than both Recent and 25-day New groups ($p<0.05$). In the deeper Layer VI, however, the Home Cage group showed elevated Zif268 compared to all three trained groups ($p<0.01$), but there were no differences in the intermediate layers.

Figure 3 shows Zif268 expression in the ILt, hippocampus and retrosplenial cortex in the four groups. In the ILt, rats in the Home Cage group expressed less Zif268 than all three trained groups (Group main effect, $F(3,25)=5.36$, $p=0.005$), but the trained groups did not differ from each other ($p>0.5$). There was no Group by ILt sub-region interaction ($F(3,25)=1.20$, $p=0.32$). Zif268 expression across the dorsal and ventral CA1 did not differ between the trained groups or the Home Cage group (Group main effect, $F(3,27)=1.39$, $p=0.26$). There was also no Group by Dorsal/Ventral interaction ($F(3,27)=1.64$, $p=0.19$). Home Cage rats did, however, express much higher Zif268 levels across both the dorsal and ventral CA3 (Group main effect, $F(3,26)=44.12$, $p<0.001$), while the three trained

groups did not differ from each other (N-K $p>0.1$).There was no Group by Dorsal/Ventral interaction ($F(3,26)=1.44$, $p=0.25$).

Interestingly, experiencing a new odor-object association at day 25 post-training resulted in lower Zif268 expression across the retrosplenial cortex (Group main effect, $F(3,25)=10.85$, $p<0.001$; N-K, $p\leq0.001$). This effect was driven by lower expression in the superficial layer for rats in the 25-day New learning group compared to all other groups (Group x Layer interaction, $F(3,25)=18.18$, $p<0.001$; superficial layer for 25-day New versus Recent group, Remote group, and Home Cage, $p<0.001$; no significant differences for deep layers). There was no Group X Sub-region X Layer interaction ($F6,50)=0.37$, $p=0.89$).

4. Discussion

The current study builds on evidence that the RE makes an important contribution to consolidation and remote recall for spatial memory (Klein et al., 2019; Loureiro et al., 2012) and contextual fear memory (Quet, Majchrzak, et al., 2020), but conflicts with evidence that the RE is not relevant for non-spatial associative memory (Quet, Cassel, et al., 2020). The current study asked whether the RE contributes to the persistence of a different non-spatial associative memory task, which engages the hippocampus during acquisition due to the inclusion of a temporal gap inserted between experience of the object and odor stimuli (Kesner et al., 2005). Our study demonstrated selectively increased Zif268 activation of RE neurons during remote recall of this non-spatial paired-associate memory task. There was a large effect size for expression in the RE relative to that shown by rats retested 5 days after acquisition, and compared to two controls groups (25-day retesting but with a new association; and a home cage control group). The paired-associate task offers the opportunity to explore memory for an association between arbitrary non-spatial stimuli, which is a core feature of the amnesic syndrome (Turriziani et al., 2004). This evidence suggests that the RE is involved in consolidation or at least retrieval of a remote non-spatial memory so task characteristics between the current odor-object association task and the social-olfactory memory task used by Quet and colleagues may explain the different findings (Quet, Cassel, et al., 2020; Quet, Majchrzak, et al., 2020). Without evidence of RE activation when there is no temporal gap between the odor and object stimuli, we cannot be sure that the critical feature is engagement of the hippocampus during learning, but this is a possibility. The engagement of the RE may be a critical factor during acquisition and performance of the odor-object paired-associate task. Compared to home cage controls, all three trained groups showed a marked increase in Zif268 at the time of testing in the current study. There was only weak evidence that the RH was engaged in a similar fashion for remote recall, although a broadly similar pattern of IEG activation was found across the three trained groups.

Evidence for increased IEG activity in the superficial layer in the medial PFC with remote recall in our non-spatial task, using Zif268 as a neuronal marker, is also consistent with previous evidence using c-Fos as a neuronal marker in spatial tasks. Remote recall in a spatial water maze task was accompanied by increased c-Fos expression in the ACC, which was attenuated by RE lesions (Klein et al., 2019). Thus, communication between this ventral midline thalamic nucleus and the medial prefrontal cortex could be critical for remote retrieval more generally beyond spatial memory. This broadens the role of the RE in hippocampal-diencephalic-cortical connectivity for consolidation and persistence across different kinds of memory, at least for tasks that are hippocampal dependent for initial acquisition.

The restrosplenial cortex and the ILt may also be involved when performing the odor-object associate memory task. The superficial layer of the retrosplenial cortex showed a reduction in activation in the 25-day New group compared to the two recall groups and the home cage controls. All three learning groups showed similarly increased Zif268 activation in both paracentral and centromedial sub-regions of the ILt compared to rats in the Home Cage group. By contrast, remote memory recall, but not recent recall, is impaired following injury to the ILt when tested on the spatial water maze task (Lopez et al., 2009). It is possible that the ILt are recruited differently across these two tasks.

The current study adds new evidence that the RE is preferentially involved in remote recall, relative to recent recall, of a non-spatial odor-object association memory. Evidence that inactivation of the RE at the time of remote recall at least in some tasks does not affect performance suggests that the RE is more relevant for the process of memory consolidation than retrieval *per se*, (Loureiro et al., 2012; Quet, Majchrzak, et al., 2020). Our experiment was not designed to answer that specific issue. The RE, however, appears to be one neural region among a growing number of thalamic sites that are involved in long-term consolidation and recall. There is evidence that disengagement of the anterodorsal thalamic nuclei facilitates remote contextual fear memory recall (Vetere et al., 2021), whereas activation of the anteromedial thalamic nuclei and ILt are relevant for remote memory of differentially-reinforced (salient) contexts and spatial cues, respectively (Lopez et al., 2009; Toader et al., 2023). Unfortunately, we had minimal Zif268 staining in the anterior thalamic nuclei in our material, so we cannot address their involvement in our study. If several nuclei contribute to consolidation and recall of a remotely acquired memory, it is possible that the loss of RE activity at the time of retrieval, for example, is not always a critical factor. This could explain evidence that environmental enrichment reinstates remote recall and elevated medial PFC activation in rats with RE lesions (Ali et al., 2017). Similarly, the failure of RE lesions to impair remote recall of social-olfactory memory may be due to the relative importance of alternate thalamic structures for that memory (Quet, Cassel, et al., 2020). Future work is needed to determine how multiple neural circuits that engage the midline thalamus cooperate in the process of both consolidation and long-term memory retrieval for different types of events.

Data availability statement

Data will be available on request.

Author contributions

JJH and JCDA: funding, concept and design, writing and editing drafts, statistical analysis and interpretation; JJH: conducting experiment.

Acknowledgments

This research was supported by University of Canterbury equipment and research grants and Early Career support (JJH) from Brain Research New Zealand – Rangahau Roro Aotearoa and Royal Society of New Zealand, Canterbury Branch.

Conflict of interest

The authors declare that the research was conducted with no conflicts of interest

5. References

Ali, M., Cholvin, T., Muller, M. A., Cosquer, B., Kelche, C., Cassel, J. C., & de Vasconcelos, A. P. (2017). Environmental enrichment enhances systems-level consolidation of a spatial memory after lesions of the ventral midline thalamus. *Neurobiology of Learning and Memory, 141*, 108-123. https://doi.org/https://doi.org/10.1016/j.nlm.2017.03.021

Dolleman-van der Weel, M. J., Griffin, A. L., Ito, H. T., Shapiro, M. L., Witter, M. P., Vertes, R. P., & Allen, T. A. (2019). The nucleus reuniens of the thalamus sits at the nexus of a hippocampus and medial prefrontal cortex circuit enabling memory and behavior. *Learning & Memory, 26*(7), 191-205. https://doi.org/https://doi.org/10.1101/lm.048389.118

Ferraris, M., Cassel, J. C., de Vasconcelos, A. P., Stephan, A., & Quilichini, P. P. (2021). The nucleus reuniens, a thalamic relay for cortico-hippocampal interaction in recent and remote memory consolidation. *Neuroscience & Biobehavioral Reviews, 125*, 339-354. https://doi.org/https://doi.org/10.1016/j.neubiorev.2021.02.025

Gilbert, P. E., & Kesner, R. P. (2002). Role of rodent hippocampus in paired-associate learning involving associations between a stimulus and a spatial location. *Behavioral Neuroscience, 116*(1), 63. https://doi.org/https://doi.org/10.1037/0735-7044.116.1.63

Hamilton, J. J., & Dalrymple-Alford, J. C. (2022). Anterior thalamic nuclei: A critical substrate for non-spatial paired-associate memory in rats. *European Journal of Neuroscience, 56*(7), 5014-5032. https://doi.org/https://doi.org/10.1111/ejn.15802

Kesner, R. P., Hunsaker, M. R., & Gilbert, P. E. (2005). The role of CA1 in the acquisition of an object-trace-odor paired associate task. *Behavioral Neuroscience, 119*(3), 781. https://doi.org/https://doi.org/10.1037/0735-7044.119.3.781

Klein, M. M., Cholvin, T., Cosquer, B., Salvadori, A., Le Mero, J., Kourouma, L., Boutillier, A. L., Pereira de Vasconcelos, A., & Cassel, J. C. (2019, 2019/05/01). Ventral midline thalamus lesion prevents persistence of new (learning-triggered) hippocampal spines, delayed neocortical spinogenesis, and spatial memory durability. *Brain Structure and Function, 224*(4), 1659-1676. https://doi.org/10.1007/s00429-019-01865-1

Lopez, J., Wolff, M., Lecourtier, L., Cosquer, B., Bontempi, B., Dalrymple-Alford, J. C., & Cassel, J. C. (2009). The Intralaminar Thalamic Nuclei Contribute to Remote Spatial Memory. *Journal of Neuroscience, 29*(10), 3302-3306. https://doi.org/https://doi.org/10.1523/JNEUROSCI.5576-08.2009

Loureiro, M., Pereira de Vasconcelos, A., Cholvin, T., Lopez, J., Merienne, N., Latreche, A., Cosquer, B., Geiger, K., Kelche, C., & Cassel, J. C. (2012). The ventral midline thalamus (reuniens and rhomboid nuclei) contributes to the persistence of spatial memory in rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience, 32*(29), 9947-9959. https://doi.org/https://doi.org/10.1523/JNEUROSCI.0410-12.2012

Mathiasen, M. L., O'Mara, S. M., & Aggleton, J. P. (2020). The anterior thalamic nuclei and nucleus reuniens: So similar but so different. *Neuroscience & Biobehavioral Reviews*. https://doi.org/https://doi.org/10.1016/j.neubiorev.2020.10.006

Quet, E., Cassel, J. C., Cosquer, B., Galloux, M., Pereira De Vasconcelos, A., & Stéphan, A. (2020). Ventral midline thalamus is not necessary for systemic consolidation of a social memory in the rat. *Brain and neuroscience advances, 4*, 2398212820939738. https://doi.org/https://doi.org/10.1177/2398212820939738

Quet, E., Majchrzak, M., Cosquer, B., Morvan, T., Wolff, M., Cassel, J. C., Pereira de Vasconcelos, A., & Stéphan, A. (2020). The reuniens and rhomboid nuclei are necessary for contextual fear memory persistence in rats. *Brain Structure and Function, 225*, 955-968. https://doi.org/https://doi.org/10.1007/s00429-020-02048-z

Ranganath, C. (2019). Time, memory, and the legacy of Howard Eichenbaum. *Hippocampus, 29*(3), 146-161. https://doi.org/https://doi.org/10.1002/hipo.23007

Silva, B. A., Burns, A. M., & Gräff, J. (2019). A cFos activation map of remote fear memory attenuation. *Psychopharmacology, 236*, 369-381. https://doi.org/https://doi.org/10.1007/s00213-018-5000-y

Toader, A. C., Regalado, J. M., Li, Y. R., Terceros, A., Yadav, N., Kumar, S., Satow, S., Hollunder, F., Bonito-Oliva, A., & Rajasethupathy, P. (2023). Anteromedial thalamus gates the selection & stabilization of long-term memories. *Cell, 186*(7), 1369-1381. e1317. https://doi.org/https://doi.org/10.1016/j.cell.2023.02.024

Turriziani, P., Fadda, L., Caltagirone, C., & Carlesimo, G. A. (2004). Recognition memory for single items and for associations in amnesic patients. *Neuropsychologia, 42*(4), 426-433. https://doi.org/https://doi.org/10.1016/j.neuropsychologia.2003.10.003

Vetere, G., Xia, F., Ramsaran, A. I., Tran, L. M., Josselyn, S. A., & Frankland, P. W. (2021). An inhibitory hippocampal–thalamic pathway modulates remote memory retrieval. *Nature Neuroscience, 24*(5), 685-693. https://doi.org/https://doi.org/10.1038/s41593-021-00819-3

Whittington, J. C., McCaffary, D., Bakermans, J. J., & Behrens, T. E. (2022). How to build a cognitive map. *Nature Neuroscience, 25*(10), 1257-1272. https://doi.org/https://doi.org/10.1038/s41593-022-01153-y

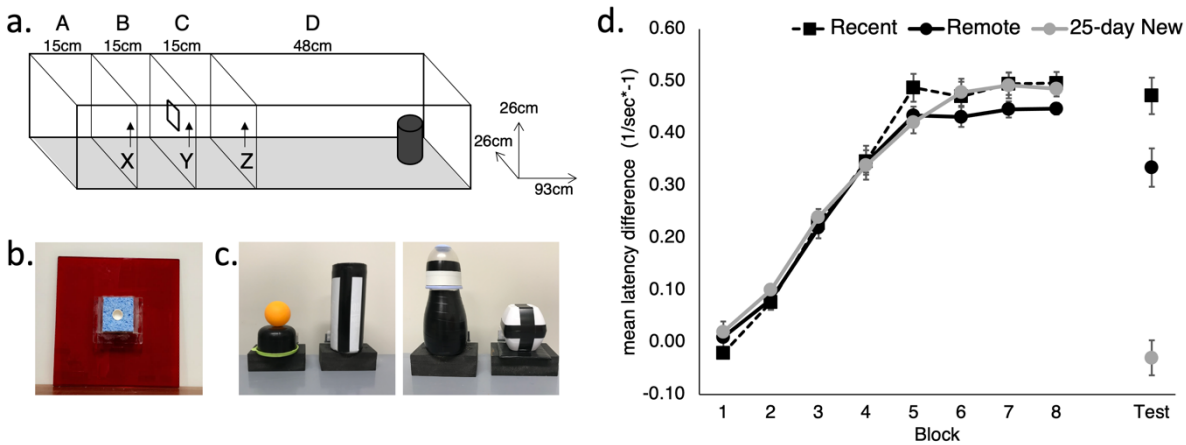

Figure 1. The odor-object paired-associate memory task. **(a)** Rats began the odor-object paired-associate memory task in Compartment A for 120 seconds on the first trial per day and 20 secs on remaining trials. Door X was removed to give access to the odour sponge **(b)** on door Y at the end of Compartment B. Door Y was immediately removed on eating the food and the rat was retained for a 10-second delay in Compartment C before Door Z was removed. Latency from removal of Door Z to interaction with the object **(c)** at the end of Compartment D was recorded. **(d)** Acquisition and retention of the odor-object paired associate task in the three groups expressed as relative latency difference between non-rewarded and rewarded pairings. The retention session was conducted 5 days (Recent) or 25 days (Remote; 25-day New) after the rat either reached criterion or the end of the acquisition period. The reciprocal latency (1/sec) was used because of the non-homogeneity of variance produced by raw latencies; the reciprocal values have been inverted by multiplying by -1. Mean +/- standard errors. Recent, N = 8; Remote, N = 8; 25-day New, N = 6.

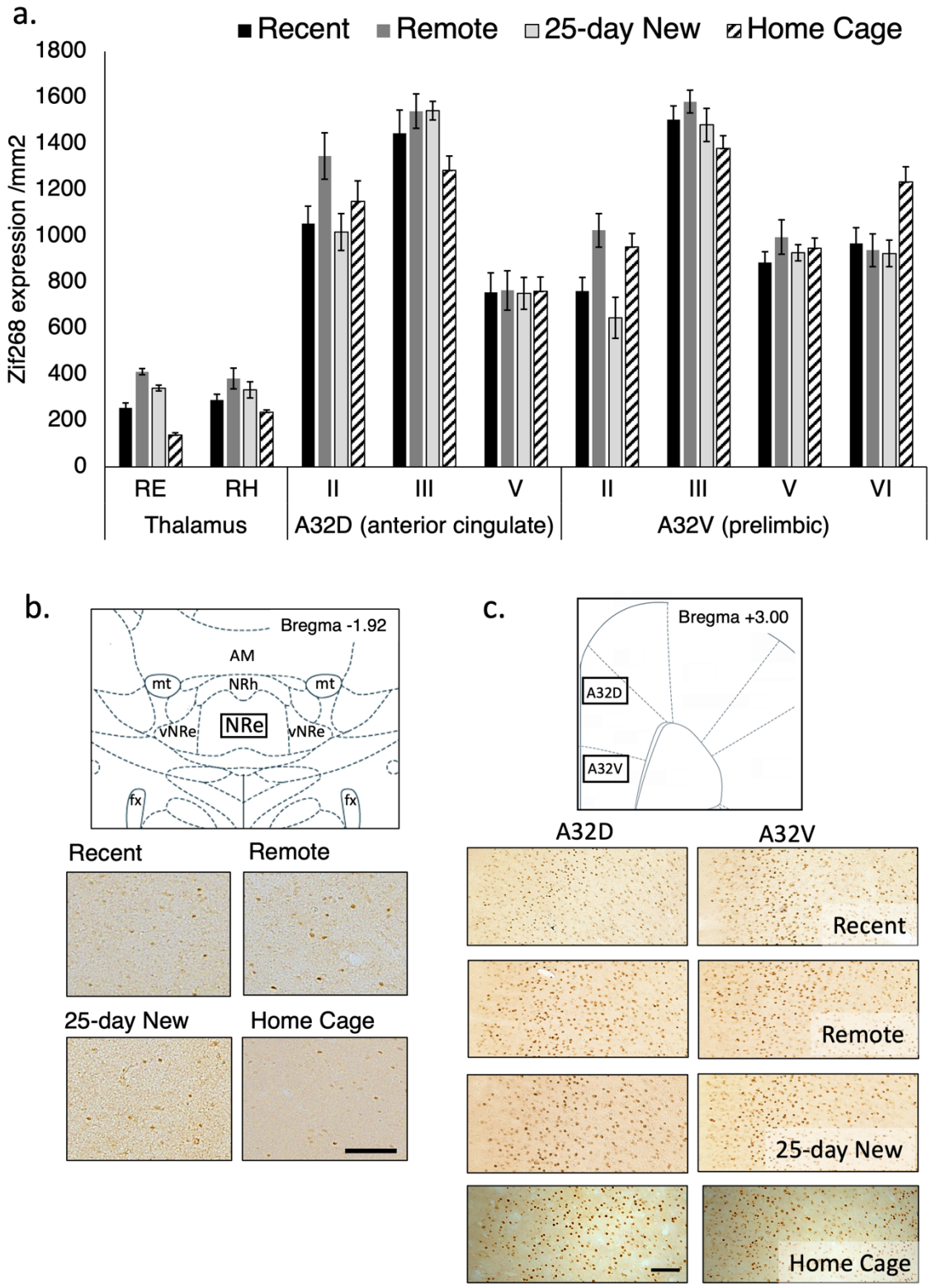

Figure 2. **(a)** Zif268 expression per mm^2 (Mean +/- standard errors) assessed in the nucleus reuniens (RE), rhomboid nucleus (RH), and prefrontal cortex regions (A32D, anterior cingulate; A32V, prelimbic) in the three groups immediately after testing at 5-days (Recent) or 25 days (Remote; 25-day New) after acquisition, as well as Home Cage controls. **(b)** Schematic diagram of -1.92 from Bregma within the RE region assessed and expression in 10x magnification photomicrographs for each group. **(c)** Schematic diagram of +3.00 from Bregma within the mPFC (A32D and A32V) regions

assessed and expression in 10x magnification photomicrographs for each group. The Home Cage control group was not trained and processed separately, but using the same Zif268 primary antibody batch number. Variation in background staining was accommodated by adjusting the background threshold in ImageJ. Recent N = 8; Remote N = 8; 25-day New N = 6; Home Cage N = 9. Black bar = 100µm.

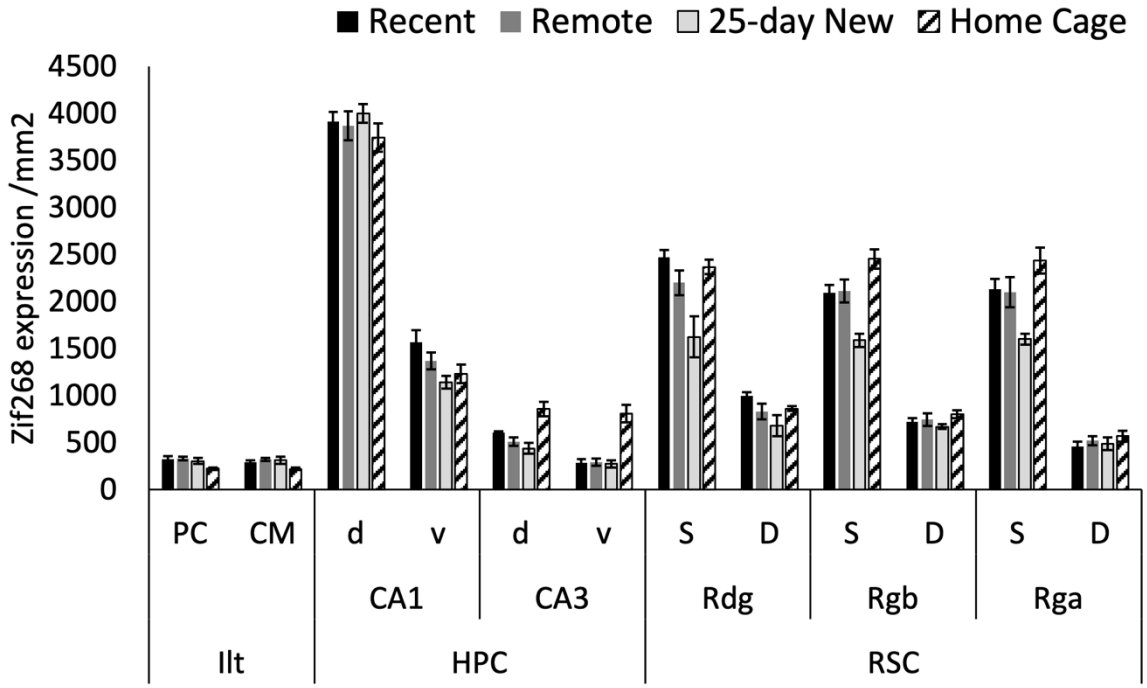

Figure 3. Zif268 expression per mm^2 (Mean +/- standard errors) assessed in the paracentral (PC) and centromedial (CM) intralaminar thalamic nuclei, dorsal (d) and ventral (v) hippocampal CA1 and CA3, and superficial (S) and deep (D) layers of three retrosplenial cortex regions (dysgranular Rdg, granular Rgb, and granular Rga) in the three trained groups and the Home Cage group. Recent N = 8; Remote N = 8; 25-day New N = 6; Home Cage N = 9.