Brief Research Report

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

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Abstract

The nucleus reuniens (NRe) in the ventral midline thalamus is ideally situated to mediate information transfer between the hippocampus (HPC) and the prefrontal cortex (PFC) and facilitate memory consolidation. As it is unclear whether the NRe influences remote memory for non-spatial associations, we trained intact adult male rats on an odour-object paired-association task in which they had to learn an arbitrary association with a 10-second lag between presentation of the stimuli. Using immediate early gene imaging (Zif268) we found clear evidence that the NRe is strongly and preferentially activated for long-term retrieval of non-spatial memory, 25 days after acquisition training. This was complemented by higher Zif268 expression in the superficial layers of the medial PFC in this group (anterior cingulate and prelimbic cortices). This increased IEG activation was found relative to a group showing recall at 5-days post-training, a control group tested at 25 days but with a new arbitrary association, as well as Home Cage controls. These findings support the conclusion that the NRe is preferentially engaged during remote, relative to recent, recall beyond spatial and fear-contextual memory to non-spatial odour-object association memory.

1. Introduction

The nucleus reuniens of the thalamus (NRe) is ideally situated to mediate information flow between the hippocampus (HPC) and the prefrontal cortex (PFC) to 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, based on lesion and inactivation studies in rats, suggests that the NRe and the closely situated rhomboid nucleus (NRh) are critical for long-term memory consolidation in some circumstances. These nuclei influence recall 25 days after acquisition, but not short-term retrieval after 5 days, in both spatial memory (Klein et al., 2019; Loureiro et al., 2012) and contextual fear memory paradigms (Quet, Majchrzak, et al., 2020). However, the relevance of the NRe for long-term memory consolidation may be limited, as it has been found not to be relevant for long-term nonspatial associative olfactory memory (Quet, Cassel, et al., 2020).

Neuronal activity based on immediate early gene (IEG) expression using the c-Fos marker also supports the involvement of NRe/NRh in remote retrieval of spatial memory (Klein et al., 2019; Loureiro et al., 2012). These studies reported elevated IEG expression for remote compared to recent retrieval in the NRe and in the anterior cingulate cortex (ACC) in the PFC (Klein et al. (2019)). One study found that the long-term retrieval of remote contextual fear memory also involved the engagement of the NRe, PFC and amygdala (Silva et al., 2019). A second study, however, reported no increase in cFos expression in the NRe at remote compared to recent retrieval of contextual fear memory (Quet, Majchrzak, et al. (2020). Together with lesion evidence, this suggests that the NRe,

perhaps in concert with the NRh, facilitates information transfer between the PFC and the hippocampal system to enable system-level consolidation and dynamic changes from a primarily hippocampal-dependent process to one that increasingly engages PFC activity. The involvement of the NRe in this relationship seems to be evident when explicit spatial information is required or when context refers to the background constellation of diffuse multimodal stimuli that represent the spatial environment.

There has been considerable debate as to whether the hippocampal system is responsible primarily for spatial memory and context or more general cognitive and relational representations. One critical issue is whether there is a temporal feature to the memory (Ranganath, 2019; Whittington et al., 2022). The question remains, then, whether the engagement of the NRe for long-term consolidation concerns spatial memory specifically or more general processes that are sensitive to hippocampal dysfunction (Quet, Cassel, et al., 2020). In rats, the acquisition of an arbitrary non-spatial association is not dependent of the hippocampus unless a temporal lag is introduced between the to-be-associated stimuli. Acquisition of an object-odour association is sensitive to hippocampal CA1 lesions when there is a 10-second lag between stimuli (Gilbert & Kesner, 2002; Kesner et al., 2005). Here, we provide evidence that the NRe shows increased IEG activation during remote, relative to recent, recall of the previously acquired object-odour association with 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. Behavioural testing occurred in the dark phase, with rats maintained at 85% of free feeding body weight and ad libitum water. All procedures complied with approved guidelines from the University of Canterbury Animal Ethics Committee. Three groups of rats were established by random assignment. Post-acquisition, one group was tested on Recent retention (5-days post-acquisition; n=8), another on 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. Paired-associate memory task

Details on the apparatus and procedures can be found in (Hamilton & Dalrymple-Alford, 2022). Training on simple odour or simple object discriminations was first used to familiarise the rats with the general procedures of a “go/no-go” procedure. Acquisition for an odour-object paired-

association was conducted in the same red Perspex runway (93x26x26cm high) with vertically removable doors (26x26cm). The odours were mixed with sunflower oil, which was placed on a sponge (6.5x6x8mm) surrounding a plastic cap at the centre of the door in a 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 odourised sponge, the rat was retained for 10 seconds in a third compartment (15cm), and then allowed to run to the end of the runway to interact with a lightweight and visually distinct object. This object was attached by a hinge to a food well underneath so the rat could, if it chose, search for a chocolate reward. The presence of food depended on whether the specific odour-object association was a "correct" trial. Black curtains enclosed the test area to minimise spatial cues. The observer sat within the curtains and adjacent to the runway to operate the doors.

Individual rats received 12 massed trials per day, six go (rewarded) and six no-go (non-rewarded) in a pseudo-randomised order, with no more than three go or no-go trials run consecutively in a session. Correct pairings for the paired-associate task were counterbalanced across rats. The critical measure was the latency to push (nose or paw) the object at the end of the runway, based on the time since opening the last door. Rats learned which of two odour-object pairings would be rewarded under the object (e.g. Odour 1 + Object A and Odour 2 + Object B) and which of two pairings were not rewarded under the object (e.g. Odour 1 + Object B and Odour 2 + Object A). A trial was terminated as 'correct' if the rat responded within 8s on go trials or withheld responding for longer than 8s on no-go trials. Latency was recorded from removal of the final door at the end of the 10s delay in the third compartment and interaction with the object or refrained from interacting with it for 8s. Rats that reached a criterion of 80% correct trials over 3 consecutive days for the task, after a minimum of 25 sessions, were then stopped from further sessions until trained again at either 5 days or 25 days later. Other rats showed clear discrimination performance task but continued testing for the maximum of 50 days (their mean difference between go and no-go trials was always > 3.2 seconds).

2.5. Post-acquisition retention test

Retention of the paired-associate task was conducted 5 days (recent recall) or 25 days (remote recall) after reaching criterion or after 50 days of training. The retention session consisted of the same previously learned association for rats in the Recent and Remote groups. For the rats in the 25-day New learning group, a completely new odour-object pairing was used for each rat, to assess the impact of a return to testing and the same 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), rats were placed in a familiar dark, quiet room for 90 min prior to perfusion. Each rat experienced this procedure for 3 days and on the third day it 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), to fix the brain. Brains were post-fixed in 4% PFA followed by a minimum of 48 h 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 until processed for immunohistochemistry.

Free-floating coronal 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, followed by incubation in biotinylated goat antirabbit secondary antibody (1:1000: Vector Laboratories BA-1000) overnight in PBSTx and 1% NGS. Following DAB visualisation (~18 min), Zif268-positive cell staining in each region of interest was photographed with a 10x objective using a light microscope (Leica, Germany). Automated counts of the cells were obtained through ImageJ (image analysis software, National Institute of Health, NIH, USA). The area surrounding regions of interest was manually selected and the images were converted to 8-bit grey scale, background was subtracted (rolling = 40), converted to mask and the watershed function was applied, and 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 in each rat to obtain the average Zif268-positive cell count per mm2.

Regions of interest were selected for their potential involvement in consolidation and recall of the odour-object paired-association. These regions included: the medial PFC (A32D, prelimbic region; ACC, A32D), HPC (dorsal and ventral CA1 and CA3), the midline thalamic NRe and NRh, 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 of rats (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 ensure homogeneity of variance. On any given test day, the transformed latencies generated a mean latency for each of the six rewarded trials and the six non-rewarded trials. Latencies were carried

forward for rats that reached criterion. The final forty days were analysed, as consistent running within the task was achieved 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 behavioural 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.

3. Results

3.1. Behaviour

The three trained groups of rats rapidly acquired both the simple odour and simple object discrimination tasks at a similar rate (data not shown; simple odour discrimination, $F(2,19)=0.08$, $p=0.92$; simple object discrimination, $F(2,19)=2.11$, $p=0.14$).

The mean reciprocal running latency for paired-associate acquisition and performance on the retention test (Recent, Remote) or performance on the new odour-object association (25-day New) is shown in Fig 1. A decline on reciprocal latency reflects an increasing latency on the non-rewarded as opposed to the rewarded trials. All three groups acquired the odour-trace-object paired associate task in a similar fashion, across the 5-day training blocks (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 in responding on the final block of acquisition (simple main effect of Group at Block 10, $F(2,19)=0.26$, $p=0.77$).

Rats in the Recent and Remote recall groups showed clear retention of the acquired association, compared to rats presented with a novel pairing (25-day New; $F(2,19)=9.63$ $p<0.001$). Rats in the two recall groups exhibited similar responding in the retention test to their last Block of acquisition (Block 10; recent or remote recall $p>0.2$), although the mean latency difference for the Remote group was marginally less than previously.

3.2 Zif268

Fig. 2 shows Zif268 expression in the NRe, NRh, and the medial prefrontal A32D (ACC) and A32V regions of interest in the three trained groups (Recent, Remote, and 25-day New) and the Home Cage controls. In the NRe, all three trained groups showed higher expression in the NRe than was found in the Home Cage group (Group main effect $F(3,25)=64.72$, $p<0.001$), suggesting that engagement in the behavioural task associates with increased activity in the NRe. Critically, N-K post hoc analysis confirmed that all four groups differed from each other on this measure in the NRe ($p<0.004$). Remote recall of the odour-object paired-associate memory was associated with

markedly higher Zif268 expression in the NRe (by a factor of 298% compared to home cage; effect size d = 7.24) compared to recent recall (185% increase compared to home cage). The standardized difference between remote recall and recent recall groups for the NRe was also a large effect, d = 4.13. Exposure to a new association pairing at 25-days after initial training produced an intermediate level of expression (247% increase compared to home cage; for the Remote group versus 25-day New group, d = 1.89).

A similar, albeit weaker, pattern of Zif268 expression to that of the NRe was apparent in the NRh (Group main effect, $F(3,25)=4.42$, $p=0.01$). However, post-hoc Newman-Keuls 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 ($p<0.10$).

There was no Group main effect for the ACC (A32D), aggregated across all three layers ($F(3,27)=1.18$, $p=0.35$). There was, however, a Group x Layer interaction ($F(6,54)=3.49$, $p=0.005$). This interaction effect was due to 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 ($p<0.12$).

Across all four layers of the A32V region of the medial PFC, Zif268 expression showed a similar pattern to that seen in the ACC. 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, but expression in this group was now significantly higher compared to both Recent ($F(1,27)=7.66$, $p=0.01$) and 25-day New groups ($F(1,27)=13.56$, $p<.002$). Home Cage rats showed an intermediate level, but higher than both Recent and 25-day New groups ($p\leq0.04$). In the deeper Layer VI, however, the Home Cage group showed elevated Zif268 compared to all three trained groups ($p\leq0.006$).

Fig 3 shows Zif268 expression in the HPC and retrosplenial cortex in all four groups. 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 x 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 behavioural groups did not differ from each other (Newman-Keuls $p>0.1$).There was no Group x Dorsal/Ventral interaction ($F(3,26)=1.44$, $p=0.25$).

Learning a new odour-object association resulted in lower Zif268 expression across the retrosplenial cortex (Group main effect, $F(3,25)=10.85$, $p<0.001$; Newman-Keuls, $p\leq0.001$). This group difference was due to significantly 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; simple main effects, superficial layer for 25-day New versus Recent and Remote groups and Home Cage, p<0.001). There was no Group x Region X Layer interaction (F6,50)=0.37, p=0.89).

4. Discussion

The current study builds on evidence that the NRe is essential 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 may not be relevant for not non-spatial olfactory associative memory (Quet, Cassel, et al., 2020). The current study addressed the possibility that the NRe may contribute to the persistence of a different non-spatial associative memory task that engages the hippocampus during acquisition due to the inclusion of a temporal gap presented between two non-spatial stimuli (Kesner et al., 2005). Our study demonstrated selectively increased activation of NRe neurons during remote recall of an object-odour non-spatial paired-associate memory. Remote recall of a this non-spatial memory resulted in a large increase in Zif268 expression in the NRe, relative to expression 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 case control group). The paired-association task offers the opportunity to explore non-spatial association of arbitrary stimuli, which is a core feature of the amnesic syndrome (Turriziani et al., 2004). This evidence suggests that the NRe is involved in consolidation and/or remote retrieval of a non-spatial memory so task characteristics between the current odour-object association task and the olfactory memory task used by Quet and colleagues may explain the different findings (Quet, Cassel, et al., 2020; Quet, Majchrzak, et al., 2020). The engagement of the NRe may also be a critical factor during acquisition of the 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 NRh 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 an increase in 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 of a spatial memory in the water maze which was accompanied by increased c-Fos expression in the ACC, which was attenuated by NRe 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 than just for spatial memory. This broadens the role of the NRe 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.

Investigation of IEGs across the extended memory network revealed that exposure to a novel association at 25-days reduced Zif268 expression in the retrosplenial cortex when compared to both retention groups. This suggests that the retrosplenial cortex, more specifically the superficial layer, could be involved in the recall of this non-spatial paired-associate task, irrespective of recent or remote consolidation. Previous evidence would indicate that our task is hippocampal-dependent as it requires the rat to retain an arbitrary association of odour-object stimuli across a 10-second delay (Hamilton & Dalrymple-Alford, 2022; Kesner et al., 2005). We would expect to see an increase of expression within the CA1 for rats exposed to the paired-associate task. Contrary to this, in the current experiment there was no difference in brain activation in the dorsal or ventral CA1 of the HPC between the trained and Home Cage rats, but also a marked decrease in activation in the dorsal and ventral CA3 for rats trained on the odour-object task.

The current study adds new evidence that the NRe is preferentially engaged during remote recall, relative to recent recall, of a non-spatial odour-object association memory. The NRe may, however, be one among a number of thalamic sites involved in remote recall. There is recent 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 is relevant for remote memory for differentially-reinforced contexts (Toader et al., 2023). Together, this evidence suggests that several midline thalamic nuclei support consolidation and / or remote memory recall. Nonetheless, there is some evidence that inactivation of the NRe at the time of remote recall may not hinder recall, which suggests that the NRe is itself specific for the process of memory consolidation rather than retrieval per se (Loureiro et al., 2012; Quet, Majchrzak, et al., 2020). Our experiment was not designed to answer this issue. If several nuclei contribute to consolidation and recall of a remotely acquired memory, it is possible that the loss of NRe activity at the time of retrieval, for example, is not a critical factor. 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 recall.

5. References

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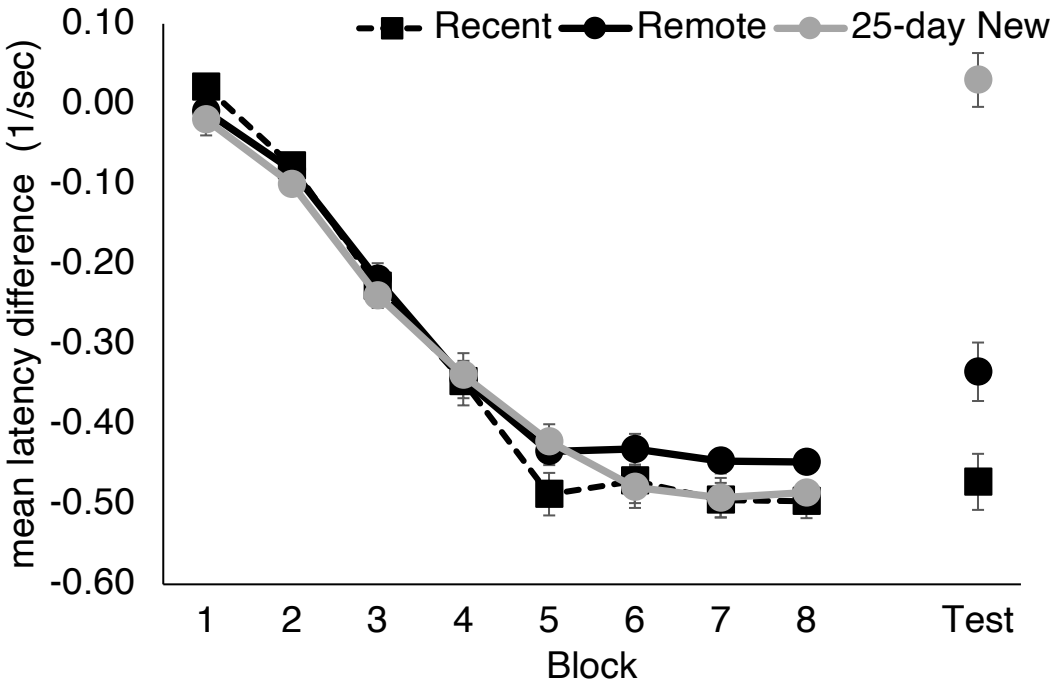

Figure 1. Odour-object paired associate task acquisition and retention expressed as relative latency difference between non-rewarded and rewarded pairings for the three groups. The retention session was conducted 5 days (Recent) or 25 days (Remote; 25-day New) after the rat either reached criterion or the end of the acquisition period. The reciprocal latency (1/sec) was used because of the non-homogeneity of variance produced by raw latencies. Mean +/- standard errors. Recent N = 8; Remote N = 8; 25-day New N = 6.

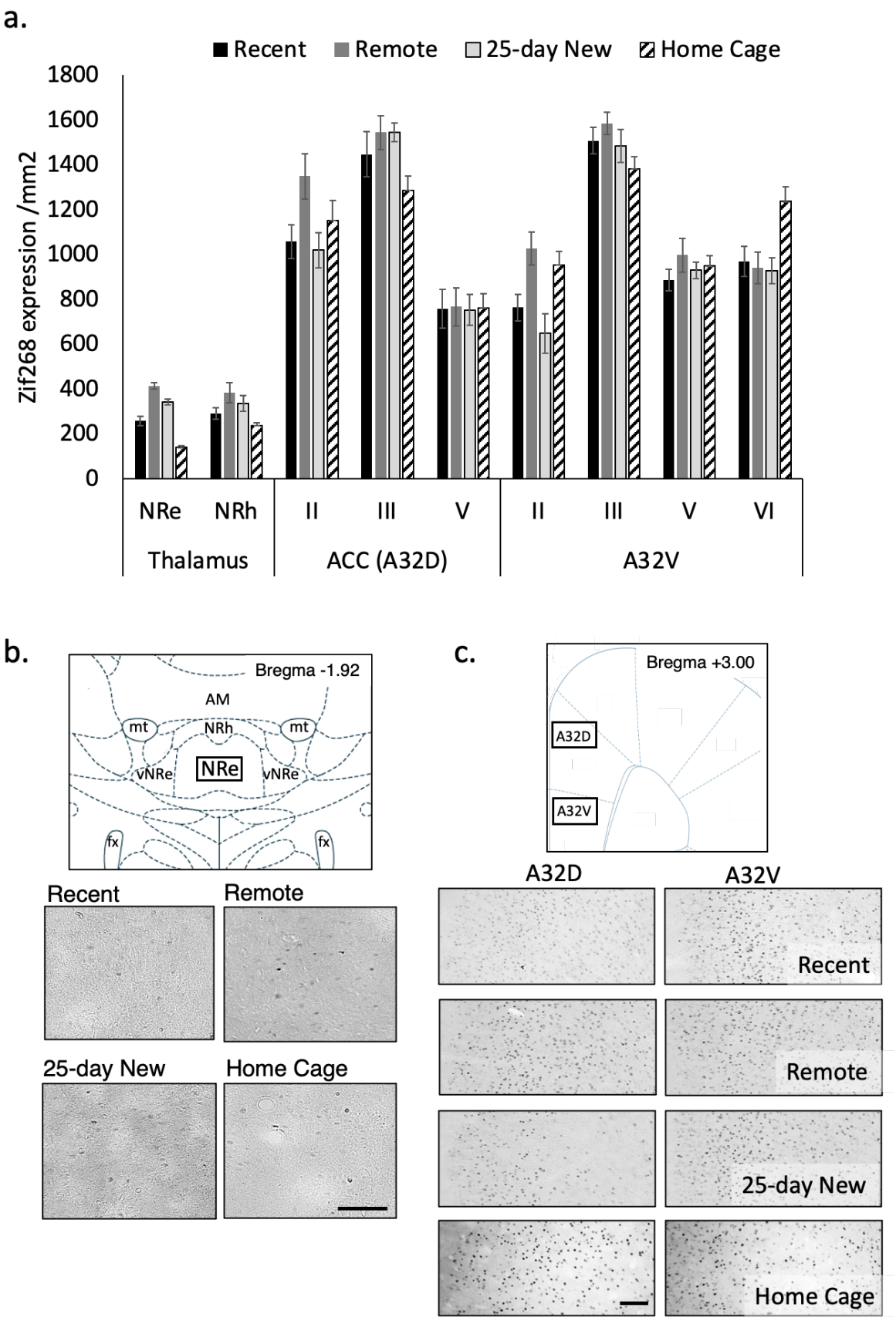

Figure 2. Zif268 expression per mm2 (Mean +/- standard errors) assessed in the nucleus reuniens (NRe) and prefrontal cortex regions (A32D and A32V) in the three groups after the 5-day or 25-day

retention test, as well as Home Cage. **(b)** Schematic diagram of -1.92 from Bregma within the NRe region assessed and mean 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 mean expression in 10x magnification photomicrographs for each group. Recent N = 8; Remote N = 8; 25-day New N = 6; Home Cage N = 9. Black bar = 100µm.

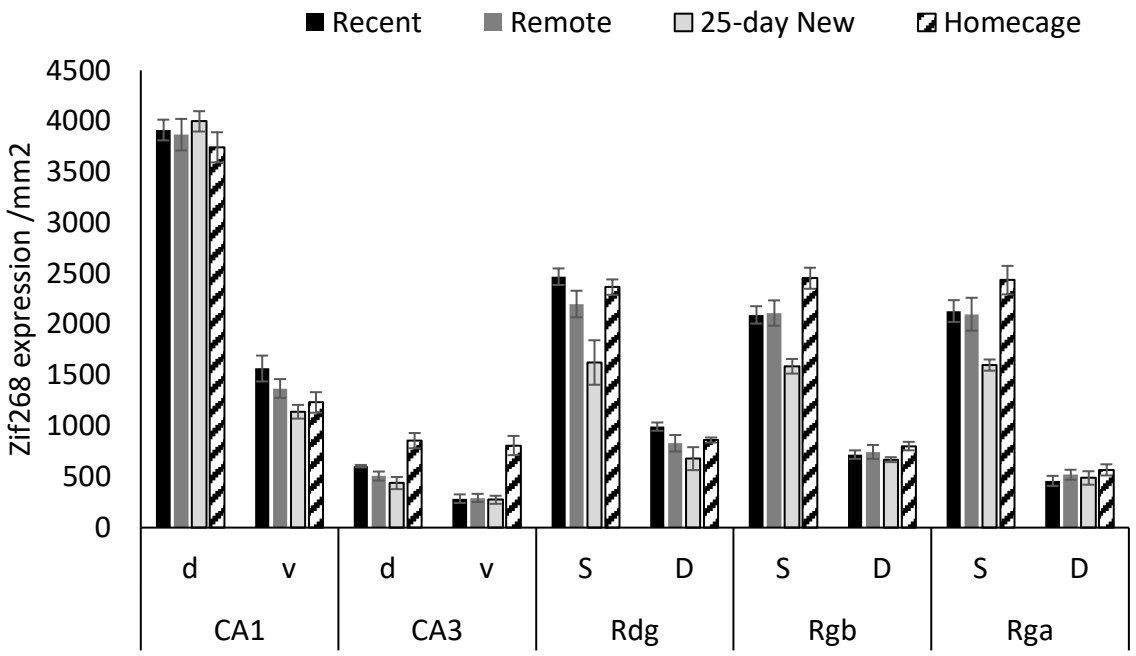

Figure 3. Zif268 expression per mm2 (Mean +/- standard errors) assessed in the dorsal and ventral hippocampal CA1 and CA3 and three retrosplenial cortex regions (dysgranular Rdg, granular Rgb, and granular Rga) in the three groups after the 5-day or 25-day retention test, as well as Home Cage. Recent N = 8; Remote N = 8; 25-day New N = 6; Home Cage N = 9.