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Neurobiology of spontaneous object exploration in recognition memory

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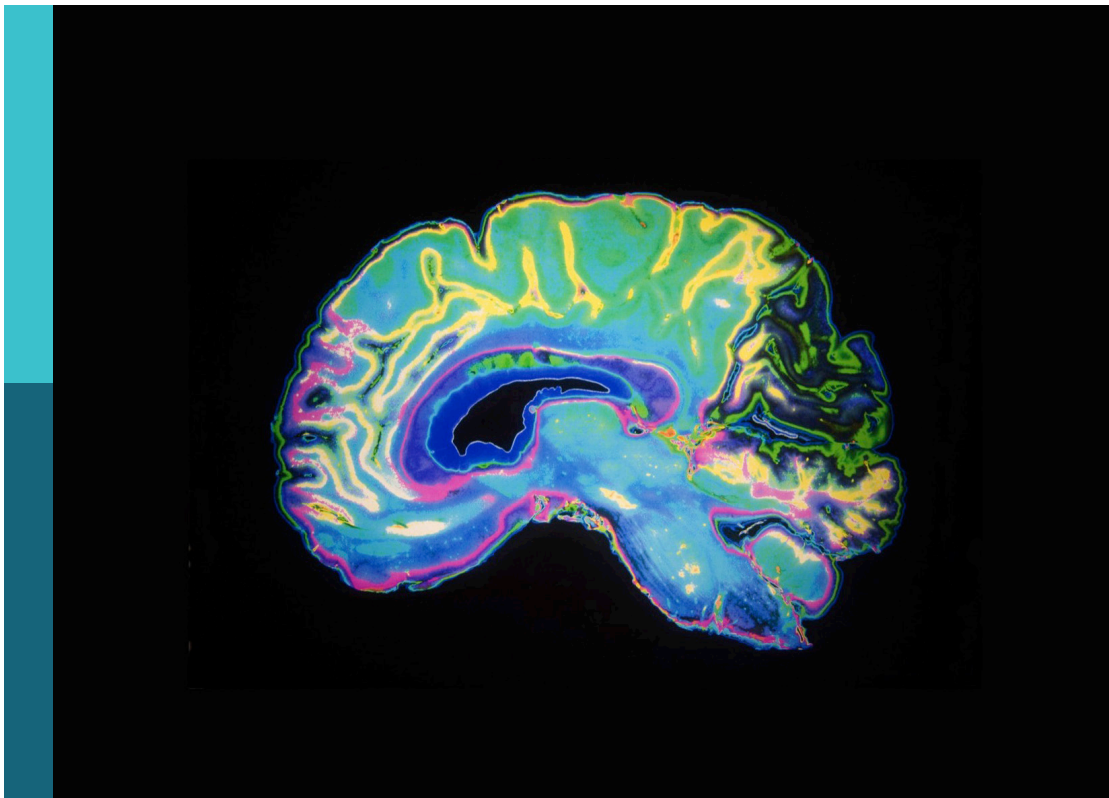

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Neurobiology of spontaneous object exploration in recognition memory

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Editorial: Neurobiology of spontaneous object exploration in recognition memory

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KEYWORDS

object recognition, episodic memory, protein kinase Mζ (PKMζ), dopamine, BDNF

Editorial on the Research Topic
Neurobiology of spontaneous object exploration in recognition memory

Spontaneous object exploration is an innate behavior that many species exhibit, which refers to the investigation of objects within the environment without explicit goals or rewards. Animals engage naturally in exploratory behaviors toward objects with novel features, such as new identity, location, and contextual associations, as compared to objects with familiar ones. This suggests that the propensity to objects with novel features is influenced by cognitive processes of perception, attention, and learning and memory, as the novelty preference is driven by remembrance of the familiar objects. Researchers have utilized spontaneous object exploration, which involves no extensive training, incentives or deterrents, and one-trial learning and retrieval, to develop paradigms for studying recognition memory of objects, places, contexts, their associations, and even episodic-like memory, mimicking human daily experiences. While significant evidence has been accumulated over the past two decades regarding the neural mechanisms of recognition memory, questions remain: How animals develop the ability to remember and distinguish between different mnemonic features of objects? Does sex play a role in recognition memory? How do distinct neurochemical systems influence recognition memory? Essentially, what are the cellular and molecular mechanisms involved in memory processing within the hippocampus (HPC) and perirhinal cortex (PRC), the two key regions for recognition memory?

We are delighted to present the Research Topic "*Neurobiology of spontaneous object exploration in recognition memory*," published in the journal *Frontiers in Behavioral Neuroscience*. This Research Topic covers recent advancements in the neurochemical, cellular, and molecular mechanisms that underlie recognition memory, with the aim of addressing important questions mentioned. The Research Topic comprises nine research papers and three review articles that explore a wide range of studies, from development to gene mutation, showcasing the diversity in this field.

How do animals acquire the ability to differentiate and memorize various features of objects? The research paper, by Asiminas et al. addresses this issue. The authors found that rats were able to form memory for objects as early as 4 weeks old, for contexts at 5 weeks, and for object-location-context association at 7 weeks. Importantly, a similar developmental

trajectory of these memories was identified among three rat strains (Lister Hooded, Long Evans, and Sprague Dawley). These findings implicate that the PRC, lateral entorhinal cortex, and medial prefrontal cortex mature around the 4th, 5th, and 7th postnatal weeks to support object, object-context and object-location-context memories, respectively.

Does sex play a role in recognition memory? The review article, by Becegato and Silva, attempts to answer this question. While female rats are often excluded from object recognition studies due to concerns related to estrous cycle, and may be subject to biases of inadequate memory capabilities, the authors suggest that male and female rats show comparable performance in most object recognition tests.

How does information load interact with schema and episodic memories? The research paper by Harkotte et al. investigates the influence of information load on the formation of schema and episodic memories. In the elaborated version of the object-place recognition paradigm, rats were asked to learn either a low or high information load of objects and places. Rats that underwent the high information load had better schema memory for the spatial rule, while those that learned the low information load had better memory for individual episodes. The contrasting outcomes could indicate a competitive relationship between schema and episodic memory formation dependent upon the encoded information load.

How do different neurochemical systems modulate recognition memory? One review article authored by Okada et al. describes that object identity and location memories are associated with the cholinergic circuits of the nucleus basalis magnocellularis-cerebral cortices and the medial spetum/ventral diagonal band of Broca-HPC/parahippocampus, respectively, which might underlie cholinergic pathology in dementia. Meanwhile, the other review article authored by Osorio-Gómez et al. discusses the role of dopamine (DA) in recognition memory, with the suggestion that DA regulates plasticity-related mechanisms that facilitate memory consolidation and persistence, thereby enhancing perceptual salience in recognition memory, regardless of the initial sensory perception. DA also modulates memory consolidation in the rat anterior retrosplenial cortex (aRSC), as indicated by the research paper of de Landeta et al. Post-sample infusions of SCH23390, a DA D1/5 receptors antagonist, into the aRSC, or that of muscimol, a $GABA_A$ receptors agonist, into the ventral tegmental area (VTA), induced object memory deficits tested 24 h later. The VTA-muscimol effects can be counteracted by aRSC infusions of SKF38393, a DA D1/5 receptors agonist. Thus, VTA might modulate object memory consolidation through the aRSC DA D1/5 receptors.

How do the cellular mechanisms of the HPC respond to the spatial properties of objects? The research paper, by Neves et al. records electrophysiological responses of the hippocampal dentate gyrus (DG), CA1, and CA3 in freely moving rats during short- or long-distance exploration between objects. Object exploration itself was linked to theta oscillations (6–12 Hz) in all the regions. Long-distance object exploration elicited higher theta power and theta-gamma phase coupling in the DG compared to exploration between neighbored objects. Stationary object exploration produced higher theta power in CA3, which correlated with CA1 gamma power. Hippocampal theta and gamma oscillations may underlie the spatial discrimination of objects into memory processing.

How do the molecular mechanisms of the HPC and PRC regulate recognition memory? The research papers, by Outram et al. and by Augereau et al. study the role of protein kinase Mζ (PKMζ) in the rat PRC concerning object memory maintenance. Post-sample (1 day, but not 6 days, later) infusions of a zeta inhibitory peptide (ZIP) that inhibits the activity of PKMζ into the PRC disrupted memory for discriminating objects, but not their places. The infusions of ZIP into the HPC produced opposite effects. Additionally, PRC ZIP infusions did not influence the perceptual ability of sensing different objects. Furthermore, blocking AMPA receptors endocytosis reversed the effects of ZIP infused into the PRC. The impairment of the long-term potential mechanism by ZIP could account for the PRC-dependent object memory maintenance. In addition, Girado et al. evaluates how the PRC endocytosis and brain derived neurotrophic factor (BDNF) affect object memory consolidation. Post-sample PRC infusions of a dynamin endocytosis function-blocking peptide disrupted object memory tested 24 h later when similar, but not dissimilar, objects were presented. Similar effects were shown when a TrkB (BDNF receptor) antagonist, ANA-12, was infused into the PRC before the learning trial. Moreover, the impairment induced by endocytosis blocking can be neutralized by BDNF infusions into the PRC. Lastly, a functional interaction effect was found between endocytosis and BDNF using a disconnection approach targeting the PRC. The PRC endocytosis interfaces with BDNF in terms of memory consolidation on similar objects.

Rossato et al. also examines the hippocampal role of c-Jun N-terminal kinases (JNK) that phosphorylates the transcription factor c-Jun in association with stress and memory in object memory consolidation and reconsolidation. Infusions of the JNK inhibitor SP600125 into CA1, 5 min, but not 6 h, after the training impaired object recognition memory. JNK inhibition did not affect the fear memory assessed by the step-down avoidance inhibitory task. The SP600125 effects were similarly shown in the reconsolidation test. Hippocampal JNK activity is important for the processes of object consolidation and reconsolidation in a time-dependent manner.

Does mutation of a disorder-relevant gene affect recognition memory? Pinizzotto et al. explore the behavioral phenotype of rats with a knockout of the phosphatase and tensin homolog-induced putative kinase 1 gene (*Pink1*) that associates with Parkinson's disease. The *Pink1* knockout rats consistently exhibited deficits in novel object, object place and object-in-place memories across ages (most cases after 5 months old). Importantly, these cognitive and memory impairments can precede the onset of confounding factors of affect and motor disturbances. The *Pink1* knockout rats could serve as a tool for investigating the cognitive neuropathology of Parkinson's disease and other neurodegenerative disorders.

This Research Topic addresses fundamental questions about the role of neurodevelopment, sex, and distinct neurochemical systems, cellular, and molecular mechanisms underlying recognition memory in the HPC and PRC. For example, memory consolidation within the PRC depends on the interaction between BDNF and endocytosis, whereas PKMζ and AMPA receptor endocytosis are associated with long-term

memory maintenance. Future experiments should explore the integrated relationship between neurochemical, cellular, and molecular mechanisms across neurodevelopment, sex, and memory stages. For instance, in memory consolidation, understanding how HPC and PRC oscillations interact with dopamine, protein kinases, and endocytosis would be valuable. Additionally, single-cell sequencing of activated neuronal populations (conceptually the engram cells) during spontaneous object exploration paradigms could establish cellular and molecular mechanisms underlying recognition memory. Ultimately, this Research Topic aims to inspire new investigations and encourage collaboration to advance our knowledge of recognition memory and its significance in health and disease.

Author contributions

OC wrote the main editorial. FB, MI, JA, and J-SL modified it. All authors contributed to research papers and review articles invitation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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Dopamine D1/D5 Receptors in the Retrosplenial Cortex Are Necessary to Consolidate Object Recognition Memory

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The retrosplenial cortex (RSC) has been widely related to spatial and contextual memory. However, we recently demonstrated that the anterior part of the RSC (aRSC) is required for object recognition (OR) memory consolidation. In this study, we aimed to analyze the requirement of dopaminergic inputs into the aRSC for OR memory consolidation in male rats. We observed amnesia at 24-h long-term memory when we infused SCH23390, a D1/D5 dopamine receptors antagonist, into aRSC immediately after OR training session. However, the same infusion had no effect on OR short-term memory. Then, we analyzed whether the ventral tegmental area (VTA) is necessary for OR consolidation. VTA inactivation by intra-VTA administration of muscimol, a $GABA_A$ agonist, immediately after an OR training session induced amnesia when animals were tested at 24 h. Moreover, we observed that this VTA inactivation-induced amnesia was reversed by the simultaneous intra-aRSC delivery of SKF38393, a D1/D5 receptor agonist. Altogether, our results suggest that VTA dopaminergic inputs to aRSC play an important modulatory role in OR memory consolidation.

Keywords: dopamine, long-term memory, SCH23390, SKF38393, posterior cingulate cortex

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INTRODUCTION

Recognition memory refers to the recall and awareness of a familiar event, individual, item, or place, allowing animals to discriminate between novel and familiar stimuli. In particular, the object recognition (OR) task has been widely used for studying the "what" component of recognition memory. The anterior retrosplenial cortex (aRSC) was recently observed to participate in OR memory consolidation (de Landeta et al., 2020), i.e., the storage of the "what" component of recognition memory. Nevertheless, there is much to unravel about the mechanisms involved in the OR memory consolidation process.

Understanding the mechanisms involved in memory consolidation is a main topic in memory research, which is relevant to better understand some memory disorders and to analyze molecular targets related to those disorders. In particular, exposure to novel stimuli induces dopamine release from the ventral tegmental area (VTA) into the hippocampus to form long-term memory (LTM) (Lisman and Grace, 2005). Moreover, dopamine is known to regulate OR memory in the prefrontal and perirhinal cortices (Nagai et al., 2007; Balderas et al., 2013; De Bundel et al., 2013; Rossato et al., 2013). Thus, dopamine is a strong candidate for modulating OR memory consolidation in the RSC.

In this regard, the RSC receives dopaminergic projections from the VTA (Berger et al., 1985; Oades and Halliday, 1987), a structure that consists mainly of dopaminergic neurons (Morales and Margolis, 2017) and that was observed to be necessary for OR memory consolidation (Rossato et al., 2013). In addition, the RSC expresses D1/D5 receptors (Diop et al., 1988) and D1/D5 activity in the aRSC is necessary and sufficient to form a long-lasting aversive memory (Katche et al., 2013). In this scenario, we hypothesized that dopaminergic inputs from VTA to aRSC are essential for OR memory consolidation. Here, we combined pharmacological and behavioral approaches to assess the role of the dopaminergic tone in the aRSC during OR memory consolidation.

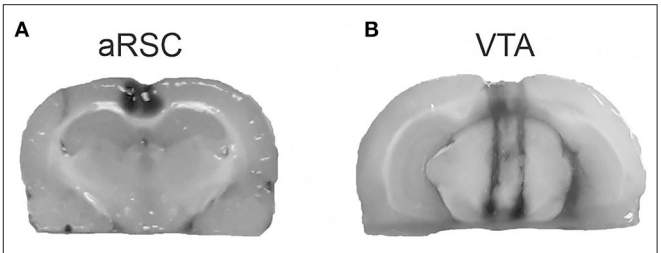

FIGURE 1 | Representation of the infusion area. Pictures show the methylene blue infusions area (black) for aRSC **(A)** and VTA **(B)**.

METHODS

Subjects

We used a total of 92 2.5-month-old male Wistar rats (Instituto de Biología Celular y Neurociencia, CONICET-UBA) weighing about 220–300 g. Animals were housed in groups of three per cage and maintained under a 12 h light/dark cycle (lights on at 7:00 a.m.) at 21–23°C with water and food *ad libitum*. Experimental procedures followed the guidelines of the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the University of Buenos Aires (CICUAL).

Surgery

Rats were implanted bilaterally under deep ketamine/xylazine anesthesia (40 and 2 mg/kg, respectively) with a 1-cm 22 G guide cannula in the aRSC at AP −3.9, L ±0.5, DV −1.8, and VTA at AP −5.3, L ±1.0, and DV −7.2, coordinates in mm from Bregma according to the atlas of Paxinos and Watson (Paxinos and Watson, 2007). Cannulas were fixed to the skull with dental acrylic. Obturators were then inserted into the cannula to prevent blockage. After 4 or 5 days of recovery from surgery, the animals were handled gently once a day for 2 days and then trained in the OR task.

Drug Infusion

To study the dopaminergic input we infused into the aRSC, the D1/D5 dopamine receptor antagonist SCH23390 hydrochloride (Sigma Aldrich, Germany) and the agonist SKF38393 hydrochloride (Sigma Aldrich, Germany) at a dose of 0.75 μg per side and 12.5 μg per side, respectively. We infused the $GABA_A$ receptor agonist muscimol (Sigma Aldrich, Germany) at a dose of 0.1 μg per side into the VTA immediately after the training session to study memory consolidation.

All drugs except SKF38393 were dissolved in sterile saline; SKF38393 was dissolved in 10% DMSO and sterile saline. Solutions used for dissolving the drugs were infused in the control group of the experiments (Vehicle, Veh). For all drugs infused, the entire infusion procedure took around 4 min, and the infusion rate was 1 μl/min. Infusions into the aRSC were 1 μl/side, while those in the VTA were of 0.5 μl/side. Injector needles were 0.1 and 0.15 cm longer than the cannula for aRSC and VTA, respectively. Injectors were left in place for an additional minute following infusion before they were removed carefully to avoid backflow.

Cannula Placement

Cannula placement was verified after the end of the behavioral procedures by infusions of 1 μl into the aRSC (**Figure 1A**) or 0.5 μl into the VTA (**Figure 1B**) of 4% methylene blue in saline. A histological examination of cannula placements was performed. Only the behavioral data from animals with the cannula located in the intended site were included in the final analysis (20 animals were excluded from the analysis).

Y-Shape Object Recognition

We performed the OR task as previously described (de Landeta et al., 2020, 2021). In brief, we habituated the animals to the empty Y-maze for 10 min, and the following day we trained the animals with two identical objects for 5 min. We then test memory 3 or 24 h after training; during the test session, we let the animals explore one object from the training session (familiar object) and one novel object for 3 min. The novel object or its position were selected by chance and were counterbalanced between animals. Objects were made of glass, metal, or plastic. The objects and apparatus were cleaned with a solution of soap, alcohol, and water before being presented to each animal.

In both training and test sessions, we used manual timers to score the time, the rodent spent exploring the objects (sniffing or touching while sniffing or facing the object). We calculated the novel object discrimination index as the exploration time of the novel object minus the exploration time of the familiar object divided by the total exploration time. Indexes significantly greater than zero were indicators of memory. We analyzed data from animals that had a minimum exploration time of 15 s/per object during the training session showing no preference for any of the sampled objects (<65% of preference for one object during training session) and that explored more than 15 s during the test (nine animals were excluded from the analysis). Total exploration times for each experiment and manipulation are shown in **Table 1**.

Data Analysis

As we used a between-subjects design for our experiments, behavioral data were analyzed using the unpaired Studen's *t*-test between groups or the theoretical value 0 and the two-way

TABLE 1 | Total training and test sessions' exploration times for each manipulation.

Figure	Group	Training		Test		dF
		Expl time (s)	*p*-value	Expl time (s)	*p*-value	
2A			0.77		0.89	12
	Veh	63.1 ± 17.2		42.7 ± 17.2		
	SCH	65.6 ± 16.0		41.4 ± 12.2		
2B			0.35		0.05	13
	Veh	73.5 ± 16.6		44.2 ± 15.2		
	SCH	84.7 ± 28.0		27.7 ± 14.6		
3A			0.12		0.16	19
	Veh	71.6 ± 12.9		41.3 ± 15.8		
	Mus	83.0 ± 19.5		32.2 ± 11.0		
3B			0.27		0.42	37
	Veh-Veh	100.4 ± 19.5		40.4 ± 16.5		
	Veh-Musc	95.7 ± 12.4		35.3 ± 16.8		
	SKF-Veh	98.3 ± 23.9		47.5 ± 18.6		
	SKF-Musc	83.9 ± 24.3		39.0 ± 13.6		

Mean ± SD exploration time for each experiment during training and test sessions. Results of two-tailed Student's t-test or ANOVA for the exploration time in each experiment.

ANOVA. We checked the normality of data using the Shapiro–Wilk test. We used Graph Pad Prism 8 (Graphpad, USA) for statistical analysis. For all analyses, the α level was set at 0.05 and the statistical power at 90% (G*Power, Universität Düsseldorf). All data are presented as mean ± SEM.

RESULTS

To analyze the requirement of the dopaminergic input for OR memory consolidation in the aRSC, we infused SCH23390 (0.75 μg/side, D1/D5 receptors antagonist) into the aRSC immediately after the training session and tested 3 h after for short-term memory (STM) or 24 h for LTM. We observed a clear-cut amnesia at 24 h in animals infused with SCH23390, while the control group had intact memory (**Figure 2A**, Studen's *t*-test. SCH vs. Vehicle: $p = 0.0042$, $t = 3.522$, $df = 12$. SCH vs. 0: $p = 0.2842$, $t = 1.176$, $df = 6$. Veh vs. 0: $p = 0.0039$, $t = 4.549$, $df = 6$. $n_{SCH} = 7$, $n_{Veh} = 7$). However, we did not observe differences in the exploration pattern between control and SCH-infused animals when testing STM, showing both groups preference for the novel object (**Figure 2B**, Studen's *t*-test. Veh vs. SCH: $p = 0.7419$, $t = 0.3365$, $df = 13$. SCH vs. 0: $p = 0.0097$, $t = 3.734$, $df = 6$. Veh vs. 0: $p = 0.0012$, $t = 5.261$, $df = 7$. $n_{SCH} = 7$, $n_{Veh} = 8$). These results show that blocking dopaminergic signaling in the aRSC prevents OR memory consolidation but not initial formation.

Next, we decided to study the possible involvement of the VTA in the dopaminergic modulation of OR LTM in aRSC. We transiently inactivated the VTA by infusing muscimol (0.1 μg/side, $GABA_A$ agonist) immediately after the training session and tested 24 h LTM. The VTA-inactivated group did not show memory, while the control group showed preference for the novel object (**Figure 3A**, Studen's *t*-test. $p = 0.0001$, $t = 4.722$, $df = 19$; Musc vs. Veh: $p = 0.0996$, $t = 1.862$, $df = 8$; Musc vs. 0: $p < 0.0001$, $t = 9.558$, $df = 11$; Veh vs. 0: $n_{Musc} = 9$, $n_{Veh} = 12$). This result indicates that VTA is required for OR memory consolidation, and it is consistent with previous results using another OR task (Rossato et al., 2013). Thus, we then studied whether this amnesia could be prevented by mimicking the dopamine input in the aRSC. We observed that the co-infusion of SKF38393 (12.5 μg/side, D1/D5 receptors agonist) into the aRSC immediately after the training session reversed the amnesic effect of muscimol-induced VTA

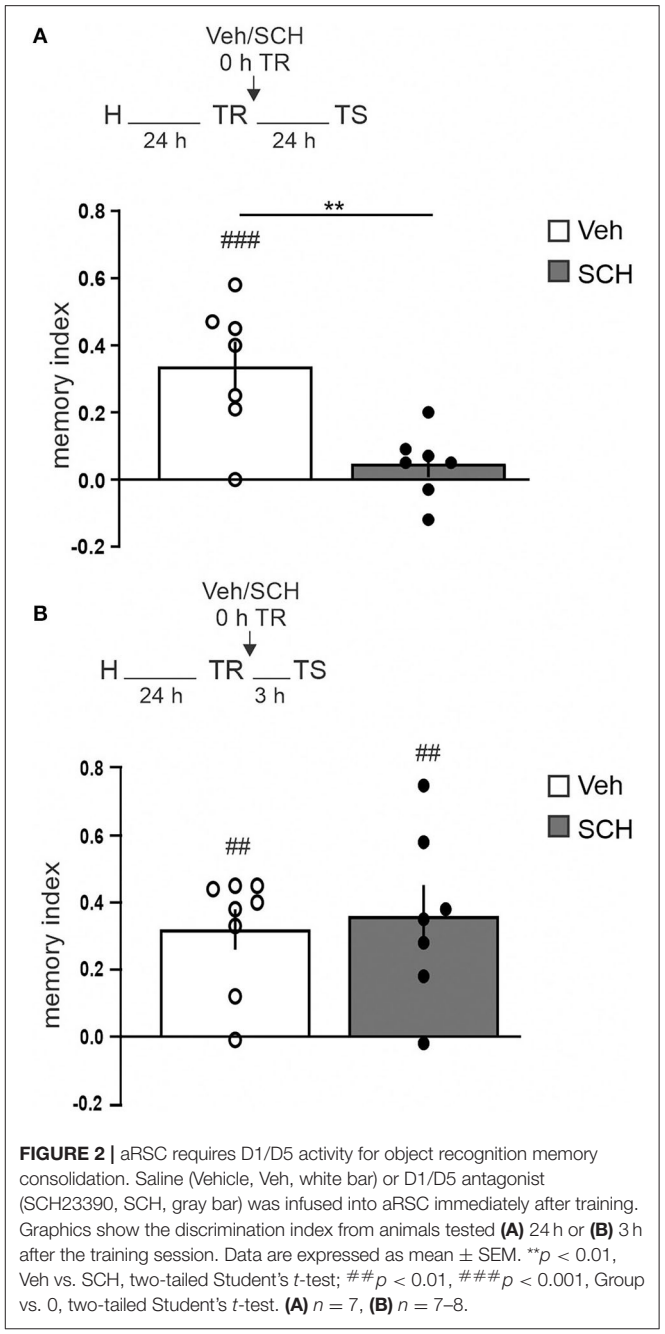

FIGURE 2 | aRSC requires D1/D5 activity for object recognition memory consolidation. Saline (Vehicle, Veh, white bar) or D1/D5 antagonist (SCH23390, SCH, gray bar) was infused into aRSC immediately after training. Graphics show the discrimination index from animals tested **(A)** 24 h or **(B)** 3 h after the training session. Data are expressed as mean ± SEM. $^{**}p < 0.01$, Veh vs. SCH, two-tailed Student's *t*-test; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, Group vs. 0, two-tailed Student's *t*-test. **(A)** $n = 7$, **(B)** $n = 7$–8.

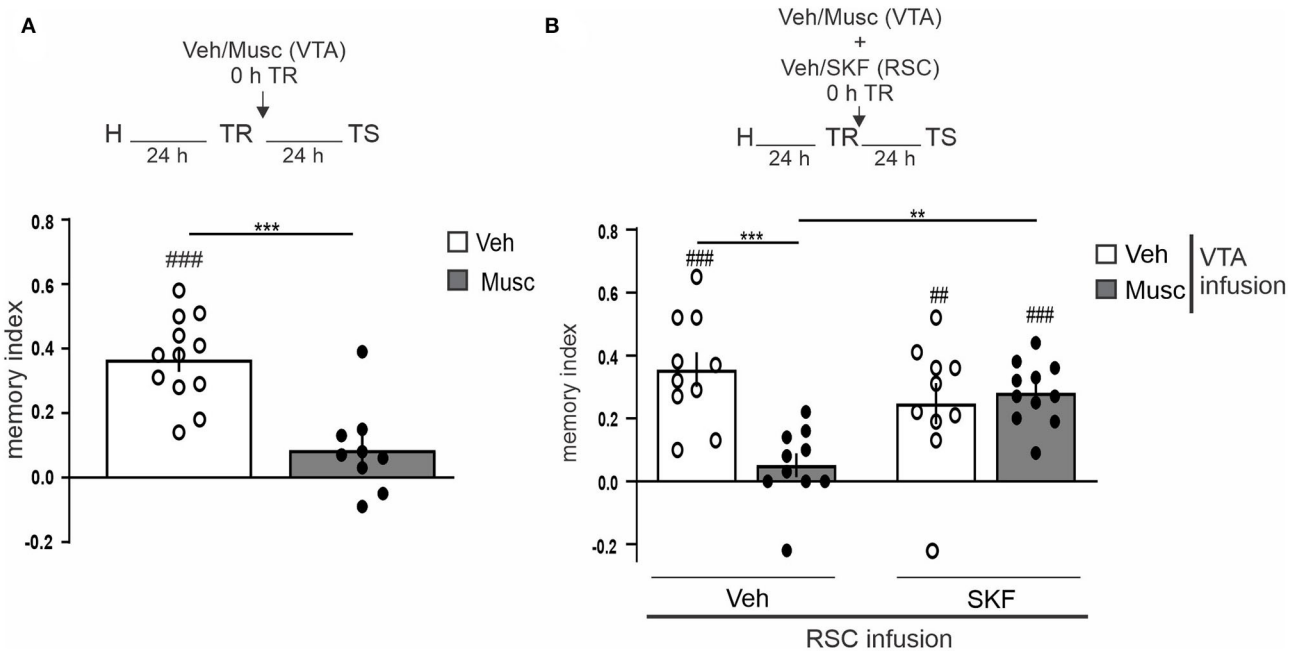

FIGURE 3 | VTA dopaminergic input to aRSC is required for object recognition memory consolidation. **(A)** Saline (Vehicle, Veh, white bar) or $GABA_A$ agonist (Muscimol, Musc, gray bar) infusions were made into VTA immediately after the training session; animals were tested 24 h after the training. **(B)** simultaneously, saline (Vehicle, Veh, white bar) or $GABA_A$ agonist (Muscimol, Musc, gray bar) was infused into VTA and 10% DMSO (Vehicle, Veh) or D1/D5 agonist (SKF38393, SKF) was infused into aRSC immediately after the training. Test session was performed 24 h after training. Data are expressed as mean discrimination index ± SEM. **(A)** $^{***}p < 0.001$, Veh vs. Musc, two-tailed Student's *t*-test. **(B)** $^{***}p < 0.001$, $^{**}p < 0.01$, Tukey's multiple comparison test after two-way ANOVA (only biologically relevant comparisons are shown). **(A,B)** $^{###}p < 0.001$, $^{##}p < 0.01$, Group vs. 0, two-tailed Student's *t*-test. **(A)** $n = 9$–12, **(B)** $n = 10$–11.

inactivation (**Figure 3B**, $p_{interaction} = 0.0012$, $F_{interaction} = 12.23$, Tukey's multiple comparisons test after two-way $ANOVA_{(1,37)}$ factors: infusion into VTA and infusion into aRSC. Studen's *t*-test: $p = 0.0001$, $t = 6.474$, $df = 9$; Veh–Veh vs. 0: $p = 0.0044$, $t = 3.768$, $df = 9$; Veh–SKF vs. 0: $p = 0.2177$, $t = 1.325$, $df = 9$; Musc–Veh vs. 0: $p < 0.0001$, $t = 9.453$, $df = 10$; Musc–SKF vs. 0: $n_{Veh-Veh} = 10$, $n_{Veh-SKF} = 10$, $n_{Musc-Veh} = 10$, $n_{Musc-SKF} = 11$). This result suggests that dopamine from VTA is not only necessary but also sufficient for OR memory consolidation in aRSC.

DISCUSSION

Our results suggest that the dopaminergic input from the VTA to the aRSC is necessary for modulating long-term OR memory consolidation. The results shown here are in line with others that showed the modulation of the dopaminergic system in OR memory by observing the enhancement of LTM when using systemic injections of dopamine D1/D5 receptor agonist SKF38393 (de Lima et al., 2011) or inhibiting the catechol-O-methyltransferase (Detrait et al., 2016). Moreover, infusion of the D1/D5 antagonist SCH23390 into the perirhinal cortex (Balderas et al., 2013), hippocampus (De Bundel et al., 2013; Furini et al., 2014; Neves et al., 2020, but see Rossato et al., 2013), amygdala (Rossato et al., 2013), or prefrontal cortex (Nagai et al., 2007; De Bundel et al., 2013; Rossato et al., 2013) produced 24 h OR amnesia, like our result when infusing SCH23390 into the aRSC. In addition, blocking dopamine reuptake in the insular cortex of an Alzheimer's disease mice model reversed the STM and LTM object amnesia in those mice (Guzmán-Ramos et al., 2012). Moreover, hippocampal dopaminergic tone is essential for object memory persistence (Neves et al., 2020; Vargas et al., 2020; Lima et al., 2022) and reconsolidation (Rossato et al., 2015; Gonzalez et al., 2021).

On the contrary, SCH23390 failed to disrupt STM formation in the medial prefrontal cortex, perirhinal cortex, and hippocampus (Savalli et al., 2015). Despite this, another study showed that the inhibition of dopaminergic activity by SCH23390 systemic administration or its infusion into the prelimbic cortex impaired OR STM (Clausen et al., 2011). Inconsistency between the results shown in these studies could be related to methodological differences, such as drug concentration and the strain of rats used. In particular, we did not find an effect of SCH23390 infusion into aRSC when testing STM. We suggest that the discrepancy between Clausen's study and ours might be due to differences in the infusion time points and in the neocortical area analyzed. In our study, we prefer to infuse SCH23390 immediately after training rather than before training; in this way, we could check whether the effect of SCH23390 on LTM was due to dopamine requirements for memory consolidation (i.e., memory stabilization) rather than deficits in acquisition or initial formation.

Although our study and others showed OR LTM impairment by SCH23390, we cannot exclude that part of this effect might be due to SCH23390 agonist activity on serotonin $5\text{-}HT_{2C}$

receptors (Millan et al., 2001). Nevertheless, it was observed that i.p. administration of a 5-HT_{2C} agonist, compound (+)-22a, improved OR LTM in a schizophrenia model (NR1-KD mice) (Cheng et al., 2016). Also, i.p. administration of the 5-HT_{2C} antagonist, RO 60-0491, enables OR LTM formation in animals that do not show LTM (Pitsikas and Sakellaridis, 2005), and the non-specific 5-HT_{2C} antagonist, agomelatine, improved the OR memory of stressed mice (Gumuslu et al., 2014), though blocking of 5-HT_{2C} receptors reinforces frontocortical dopaminergic transmission (Millan et al., 2003). Thus, 5-HT_{2C} receptor activity could be related to OR memory formation improvement. This strengthens that our results are related to SCH23390 activity over D1/D5 receptors.

The requirement of VTA for OR memory consolidation observed in this study is similar to that previously shown in another OR task (Rossato et al., 2013). In addition, our results showed that mimicking dopamine input by the simultaneous infusion of a D1/D5 agonist into the aRSC prevented the amnesia produced by VTA inactivation. Likewise, D1/D5 activity in the medial prefrontal cortex and amygdala together, but not each structure alone, could prevent the effect of VTA inactivation (Rossato et al., 2013). The main difference between Rossato's work and ours is that we observed that local SKF38393 only in the aRSC prevents the VTA inactivation effect, suggesting that the aRSC is a prime structure for OR processing. Considering its functional connectivity with many brain regions of the OR network (de Landeta et al., 2021), we suggest that aRSC could be relevant for receiving and sending information about different features of the objects, orchestrating object memory consolidation. However, when the aRSC is not properly functioning during memory acquisition, this role might be taken over by other brain structures (de Landeta et al., 2020).

Our results showed for the first time that dopamine is required in the aRSC for OR memory consolidation; we demonstrated that dopamine is both necessary and sufficient to consolidate OR memory in the aRSC. These results also suggest the involvement of VTA inputs to the OR memory network for the proper memory consolidation. Considering VTA cellular diversity and the existence of neurons that co-release dopamine and either GABA or glutamate (Morales and Margolis, 2017), we cannot conclude about the nature of VTA inputs into the aRSC and their effect on OR memory. To consolidate the link between the effect of VTA transient inactivation and D1/D5 signaling in the aRSC, further experiments are needed to selectively manipulate the VTA dopaminergic neurons projecting to the aRSC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of the University Buenos Aires (CICUAL). School of Medicine, University of Buenos Aires.

AUTHOR CONTRIBUTIONS

ABL, JHM, and CK designed the experiments and wrote the manuscript. CK led the research. ABL performed the experiments and analyzed the data. All authors contributed to the article and approved the submitted version.

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Effects of Information Load on Schema and Episodic Memory Formation

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The formation of semantic memories is assumed to result from the abstraction of general, schema-like knowledge across multiple experiences, while at the same time, episodic details from individual experiences are forgotten. Against this backdrop, our study examined the effects of information load (high vs. low) during encoding on the formation of episodic and schema memory using an elaborated version of an object-place recognition (OPR) task in rats. The task allowed for the abstraction of a spatial rule across four (low information load) or eight (high information load) encoding episodes (spaced apart by a 20 min interval) in which the rats could freely explore two objects in an open field arena. After this encoding phase, animals were left undisturbed for 24 h and then tested either for the expression of schema memory, i.e., for the spatial rule, or memory for an individual encoding episode. Rats in the high information load condition exhibited a more robust schema memory for the spatial rule than in the low information load condition. In contrast, rats in the low load condition showed more robust memory for individual learning episodes than in the high information load condition. Our findings of opposing effects might point to an information-load-dependent competitive relationship between processes of schema and episodic memory formation, although other explanations are possible.

Keywords: schema memory, episodic memory, information load, object recognition, memory tradeoff

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INTRODUCTION

Forming new memories through learning is contingent on prior knowledge (Winocur et al., 2010; Ghosh and Gilboa, 2014; Gilboa and Marlatte, 2017). For example, understanding the content of a scientific article relies on concepts or mental schemas that the reader already possesses. More formally, mental schemas can be described as higher-level knowledge structures that organize lower-level representations in long-term memory (Gilboa and Marlatte, 2017; Klinzing et al., 2019). Schemas can take different shapes, such as narratives about causal relationships, concepts, and categories in which we understand the world or knowledge about recurrent patterns. It is assumed that the formation of schema memory relies on the abstraction of experiences from single or multiple episodes into general, more abstract knowledge that lacks episodic detail also referred to as event gist (Inostroza and Born, 2013; Gilboa and Marlatte, 2017; Sekeres et al., 2018; Alonso et al., 2020). The timescale over which such abstraction occurs differs for the type of schema

memory under investigation. For instance, narratives about causal relationships can be encoded within a single episode, whereas the often implicit knowledge about recurring patterns forms over multiple episodes (Tse et al., 2007; Gilboa and Marlatte, 2017; Genzel et al., 2019). Multiple experiences generally benefit the formation of schemas, either through the assimilation of new external information into mental schemas or the adaptation of already existing schemas by taking into account new related information (Winocur et al., 2010; Gilboa and Marlatte, 2017).

In contrast to schema memory, in episodic memory, the details of a single episode are retained with high fidelity. Systems memory consolidation processes may both strengthen episodic memories and promote the transition from episodic to schema memory (Inostroza and Born, 2013; Schapiro et al., 2017). Episodic details are lost either through processes of decay or processes of interference (Sadeh et al., 2014, 2016; Polack et al., 2017; Sun et al., 2017), and recent studies in humans demonstrated that schema memory guides the recall of spatial and item memories of an experienced episode after longer retention times when their relative strengths change (Zeng et al., 2021; Ramey et al., 2022). However, it is unknown whether these processes can occur in parallel or are competing processes as they rely on similar neural structures. To date, only a few studies have examined this question. Evidence from human studies suggests that a vast amount of information during learning impedes the formation of persisting episodic memory representations (Feld et al., 2016; Feld and Born, 2017; Kolibius et al., 2021). Indeed, assuming that sleep is necessary for consolidating episodic memory, Kolibius et al. (2021) proposed that the effect of memory consolidation scales with information load due to a limited capacity available for sleep-dependent memory consolidation. Therefore, information load at learning might mediate the formation of schema and episodic memory in opposite directions, i.e., schema memory benefits from larger amounts of information whereas episodic memory becomes blurred.

Here, we aimed to test this hypothesis in adult rats using an elaborated version of the object-place recognition (OPR) task. Although many paradigms have been established to study different aspects (i.e., what, where, and when components) of episodic memory in rodents (Binder et al., 2012; Takeuchi et al., 2016; Oyanedel et al., 2019), there are only a few attempts to study the behavioral expression of schema memory. For example, using an object-place reward learning task, McKenzie et al. (2014) demonstrated that such object-place associations are hierarchically represented in hippocampal structures, presumably supporting processes of pattern separation and completion in schema memories. However, a caveat of these and similar tasks (Tse et al., 2007) is the use of emotional stimuli, positive rewards, or aversive electrical shocks, that may bias the formation of schema memory. Additionally, these tasks often do not allow for an assessment of truly episodic memory (i.e., for an event occurring in a unique spatio-temporal context) because the animals need to be trained repetitively with the same task stimuli. Hence, for contrasting the formation of schema memory and episodic memory in an unbiased manner, tasks like the OPR task might be advantageous as they exploit the rodents' natural tendency to explore novelty (Binder et al., 2012; Oyanedel et al., 2019). Findings that rats and mice are able to form a cumulative memory for a spatial rule in an adapted version of the OPR task (Genzel et al., 2019) represent the first evidence that such tasks provide a promising approach to the joint assessment of episodic and schema memory in rodents.

Accordingly, here, we used an elaborated version of the OPR task to examine the question of whether information load during learning affects the formation of episodic and schematic memory in opposite directions. The task consisted of either four (low information load) or eight (high information load) consecutive encoding episodes, in which animals explored different pairs of identical objects. To test schema memory, the objects were positioned according to a spatial rule across all episodes, and memory was assessed 24 h later by positioning the objects such that one object violated the spatial rule. Based on the rodent's natural tendency to explore novelty, we expected animals that had successfully formed a schema memory for the rule, to preferentially explore the object that violated the rule. To test episodic memory, we presented the rats also with four or eight encoding episodes, but with no spatial rule present across episodes. Memory was assessed for the last encoding episode again 24 h later, by re-exposing the animal to these objects with one object displaced to a different location. Rats that successfully formed an episodic memory were expected to preferentially explore the displaced object. We hypothesized that high information load during encoding supports schema memory formations while episodic memory is absent. Conversely, a low load of information during encoding should result in episodic memory but not schema memory.

MATERIAL AND METHODS

Animals

Forty adult male Long-Evans rats (Janvier, Le Genest-Saint-Isle, France), 9–12 weeks old at the beginning of the experiment, were used in this study. Rats were housed in groups of two-four per cage with ad libitum access to food and water throughout the experiment and were kept on a 12 h/12 h light-dark cycle (lights on at 6:00 a.m.). Before starting behavioral testing, animals were handled daily for 10–15 min on five consecutive days. All experimental procedures were performed in accordance with the European animal protection laws and policies and were approved by the Baden-Wuerttemberg state authorities.

Apparatus and Objects

An elaborated version of the object-place recognition (OPR) task was performed in a quadratic open field arena (80 × 80 × 40 cm, made of gray PVC), which was dimly lit with 20–30 lux and equipped with a masking white noise of 60 dB. A camera (Logitech C920) was mounted above the open field. The camera as well as posters affixed to the walls of the testing room and surrounding curtains represented distal spatial cues. Eight pairs of glass objects of different shapes and sizes (height 15–30 cm, bottom diameter 7–12 cm), filled with sand of different colors, were used in the experiments. To assure that rats could effectively

discriminate the different objects, only sets of objects were used that had previously been tested in experiments that used the novel object recognition task (Sawangjit et al., 2018). Objects had sufficient weight to ensure that rats could not move them. The arena and objects were cleaned after each trial with 70% ethanol solution to prevent variance in smell.

Experimental Procedures, Task, and Design

Prior to the experiments, animals were habituated to the testing room and open field arena. For that, animals were brought inside their home cage into the testing room on three consecutive days before the experiment was performed. After the animals spent at least 15 min inside the testing room they were placed inside the empty open field arena facing a different wall of the arena during each habituation session. The animals could then freely explore the arena and its surrounding cues for 10 min. Afterward, animals were brought back to their home cage and to the animal facility where they were kept.

Twenty-four hours after the last habituation session, the rats were again brought to the test room for the encoding phase of the elaborated version of the OPR task. The encoding phase comprised either eight (high information load) or four (low information load) consecutive encoding episodes separated by an inter-trial interval of 20 min. In each trial, a different pair of identical objects were placed in two out of eight possible locations in the arena, ensuring that objects were equally distant to the arena walls (10 cm from the bottom of the objects, **Figure 1**). For the schema version of the task, one location inside the arena was occupied by an object during all encoding episodes, while two other locations were occupied by an object every second episode. Thus, a spatial rule existed across encoding episodes such that one location was always occupied by an object, whereas two locations were only partially occupied across episodes. Accordingly, the spatial rule was sufficiently presented only after the animal had completed at least two encoding episodes. For the episodic memory version of the task, no such spatial rule was present across the encoding episodes. Instead, the object pairs were placed semi-randomly in two out of eight possible locations in the arena, with the constraint that all possible locations were occupied equally often across episodes (**Figure 1C**).

For all experiments, animals entered the open field facing a different wall of the arena at each encoding episode to promote the formation of an allocentric spatial representation. The duration of each encoding episode was 5 min. For practical reasons, in subgroups of six animals in each the low and high information load conditions, encoding duration was reduced to 3 min. The explorative behavior of these animals did not differ from those with 5-min episodes, $p > 0.10$ for all relevant parameters. During the inter-trial interval, animals were kept in their home cage and after completion of the encoding phase, animals were brought back into the animal facility.

Twenty-four hours after the encoding phase, animals were brought back to the test room and tested for either memory of the spatial rule or episodic memory of the last encoding episode. In the schema version of the task, the object pair used in the first encoding episode was again placed in the arena. However, this time the object that had been placed at the always occupied location during encoding was moved to a location that had never been occupied during the encoding phase, while the other object was moved to the location that was partially occupied during the encoding phase but, had not been occupied during the first encoding episode. Thus, both objects were moved to a location different from that during the encoding episode, but only the placement of one object violated the spatial rule (i.e., that one location is always occupied by an object) enabling the separate assessment of schema memory.

The episodic version of the task (not comprising a spatial rule at encoding) should provide a separate measure of episodic memory unbiased by any schema memory formation. For testing episodic memory, the object pair of the last encoding episode was again placed in the arena and, like in the classical OPR task, one of the objects was moved to a different location while the other (stationary) object remained at the same location as during encoding. We focused on the last encoding episode to exclude the effects of (retroactive) interference. Animals from both high and low information load groups were subjected to the same procedure during the test session. The duration of the test trial was 5 min for all groups.

Ten animals were randomly assigned to each experimental group, i.e., low information load/schema memory, high information load/schema memory, low information load/episodic memory, and high information load/episodic memory, according to a between-groups design. All experiments were carried out between 8:00 a.m. and 14:00 p.m. Locations in which objects were placed and the type of objects were randomized across encoding and test phases.

To assess memory performance, exploratory behavior directed towards the objects during the encoding and test trials was manually scored after the completion of all experiments using tracking software (ANY-maze, Stoelting Europe, Dublin, Ireland). Object exploration was defined as the rat being within 1 cm of an object, directing its nose towards the object, and engaging in active exploration behaviors such as sniffing. Leaning on the object without sniffing close to the object (>1 cm) was not counted as object exploration behavior. All scoring was done by the same experienced experimenter, who was blinded to the experimental condition. To assess memory retrieval for the spatial rule (schema memory) or object-place recognition memory (episodic memory) a discrimination ratio was calculated according to the general formula:

$$\frac{\text{object exploration time at novel location} - \text{object exploration time at familiar location}}{\text{object exploration time at novel location} + \text{object exploration time at familiar location}}$$

Novel location refers to the object at a previously never occupied location in the schema memory test and to the displaced object in the episodic memory test. A positive discrimination ratio indicates memory for the spatial rule or for the stationary object, respectively, whereas a value of zero indicates no exploration preference.

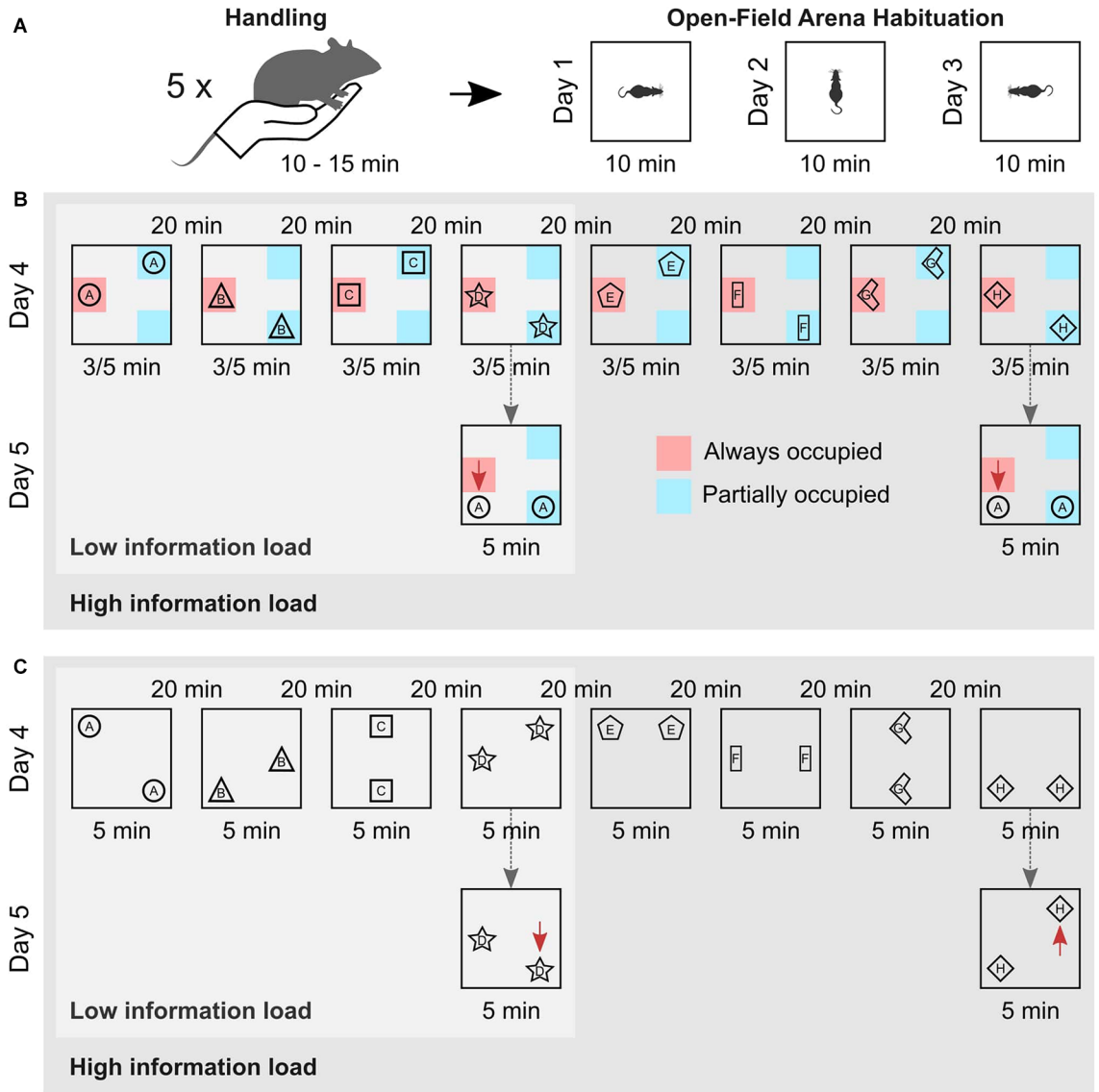

FIGURE 1 | Experimental procedure. **(A)** Animals were handled on five consecutive days for 10–15 min prior to the experiment (left). Then, they were habituated to the empty arena for 10 min on three consecutive days. They entered the arena facing different walls in each session to promote allocentric navigation (right). **(B)** Schema memory task. Animals either performed four (Low information load) or eight (High information load) encoding episodes, with an inter-trial interval of 20 min. In each episode, the rats explored different pairs of identical objects for 3/5 min, which were arranged according to a spatial rule across trials, i.e., one location was always (red zone) and two locations were only partially occupied (blue zones). Schema recognition memory (of the spatial rule) was tested 24 h later (dashed arrows). For this, objects from the first encoding episode were moved, so that both occupied locations different from encoding. The location of one object (red arrows) thus violated the spatial rule. Schema memory is assessed based on the increased exploration time the animal devotes to the object violating the spatial rule in comparison to the time spent exploring the other object. **(C)** Episodic memory task. Animals also performed on either four (Low information load) or eight (High information load) encoding episodes with different pairs of identical objects on each episode. But, objects were arranged without a spatial rule, with all locations equally often occupied by an object across episodes. Episodic memory for the last encoding episode was tested 24 h later (dashed arrows) with the objects from this episode arranged such that one object was moved to a novel position (displaced object, red arrows), while the other remained at the familiar location (stationary object). Episodic recognition memory was assessed based on the increased exploration time the animal devoted to the displaced object in comparison to the time spent exploring the stationary object.

Data Reduction and Statistical Analyses

To assess indicators of motivation and locomotion, the total object exploration time and distance traveled during encoding and test phases were extracted from the videos. This data was then further analyzed using statistical software (R, R Core Developer team). Data from individual rats were discarded, when animals exhibited consistently low exploration times during the encoding phase, specifically when rats spent <1 s exploring both objects in more than 50% of the episodes. This resulted in four rats being discarded from the dataset and a total

number of 36 rats (low information load/schema memory $n = 9$, high information load/schema memory $n = 9$, low information load/episodic memory $n = 9$, high information load/episodic memory $n = 9$) included in the final analyses.

Discrimination ratios from the test phase were calculated separately for each minute and the total 5-min duration. For statistical analyses, a mixed linear model was fitted using the lm4 package (Bates et al., 2015) with individual rats as random effect (random intercept only) and the fixed effects Information Load (High vs. Low), Task (Schema vs. Episodic) and Minute (1st vs. 5th minute of test trial):

$$DR \sim (InfoLoad * task * Minute) + (1|animal)$$

where DR indicated the discrimination ratio over the 5-min test interval. The significance of factors was assessed by removing the respective factor or interaction of two factors step by step from the model and comparing the modified models with the original using likelihood-ratio tests. In addition, control parameters including total distance traveled and total object exploration time during encoding and test trials were analyzed using the same approach. For comparisons, two-sided Welch *t*-tests were computed. Correlational analyses were based on Spearman correlation coefficients to account for the low number of animals and were compared using both, Fisher's z and Zou's confidence intervals (level of confidence: 0.95) as implemented in the corcor toolbox (Diedenhofen and Musch, 2015). For all analyses a $p < 0.05$ was considered significant. Results are reported as the means ± SEM.

RESULTS

At the test phase, 24 h after encoding, the rats exhibited significant schema memory only in the high information load condition ($\chi^2_{(1)} = 5.99$, $p = 0.014$, for the difference in discrimination ratios between high and low information load during schema memory testing, **Figure 2A**). In the high information load condition, schema memory performance above chance manifested itself after the first minute of the test phase (all $p < 0.02$) and approached significance already in the first minute ($t_{(8)} = 1.89$, $p = 0.09$). When animals performed only four learning trials in the low information condition, no memory above chance was found (all $p > 0.2$). Correlational analysis revealed that only in the high information load condition a high preference for the partially occupied location during the last encoding episode was predictive of a higher memory performance at the test ($rho = 0.75$, $p = 0.021$), but not in the low information load condition ($rho = 0.078$, $p = 0.76$, $z = 2.69$, $p = 0.007$ for difference between correlations, **Figure 2C**). These findings indicate that only in the high information load animals were able to form and retrieve schema memory for the spatial rule that was present during the encoding trials.

In contrast, on the episodic memory test, the rats exhibited an object-place memory for individual encoding episodes that was above chance, only in the low information load condition. Respective discrimination ratios were significant in the 2nd min of the test phase ($t_{(8)} = 2.70$, $p = 0.02$, **Figure 2B**) and approached significance in minutes 3–5 (all $p < 0.09$). Animals in the high information load condition did never exhibit discrimination ratios above chance (all $p > 0.2$). The difference between the low and high information load conditions across all minutes did not reach significance, however ($\chi^2_{(1)} = 2.49$, $p = 0.113$). Overall, these findings hint towards a modulating role of information load for episodic memory, with this effect, however, being weaker than on the formation of schema memory.

To address whether the effect of information load during encoding on episodic vs. schema memory formation acts in opposing directions, the discrimination ratios across all experimental groups were compared. Evidence for such an opposing effect was indeed present, as the expression of recognition memory was dependent on an interaction between information load and the type of memory assessed ($\chi^2_{(1)} = 8.04$, $p = 0.004$ for Information load × Schema/Episodic memory interaction).

To exclude that the observed effects resulted from unspecific motivational differences between the groups during encoding, the traveled distance and total object exploration time across all encoding episodes, and during the first and last episode were compared (To include all animals, this was done for the first 3 min of each episode). For the first encoding episode, neither the traveled distance nor the total object exploration time differed between groups (all $p > 0.1$), ruling out any unspecific differences (**Figure 3A**). For the last encoding episode (i.e., the fourth and eighth, respectively) the traveled distance decreased more in the high than in the low information load condition ($\chi^2_{(1)} = 6.04$, $p = 0.019$), while the total object exploration time remained comparable across groups (all $p > 0.3$, see **Figure 3B**). Indeed, the decrease in locomotion is plausible suggesting a higher level of habituation for animals that spent a greater number of episodes in the arena. In line with this finding, also the mean traveled distance across all encoding episodes was revealed to be lower in the high than low information load condition ($\chi^2_{(1)} = 5.57$, $p = 0.018$, **Figure 3C**, left panel).

Unexpectedly, mean total object exploration time across all encoding episodes depended on an interaction between information load and type of task ($\chi^2_{(1)} = 4.31$, $p = 0.037$) which was largely driven by longer mean exploration durations in animals of the high information/schema memory group (**Figure 3C**, lower panel). While this effect is difficult to explain, we excluded the possibility that the longer exploration durations in this group contaminated the observed effects of episodic vs. schema memory by running a separate mixed model analysis that controlled for the mean exploration time at sampling. This analysis confirmed the initial finding of the significant effect of information load on the formation of episodic vs. schema memory ($p = 0.00017$, for respective Information load × Schema/Episodic memory interaction).

DISCUSSION

The abstraction of gist information from multiple experiences into schema memory and the formation of detailed episodic memory from individual experiences serve different functions for the mammalian memory system (Wang and Morris, 2010).

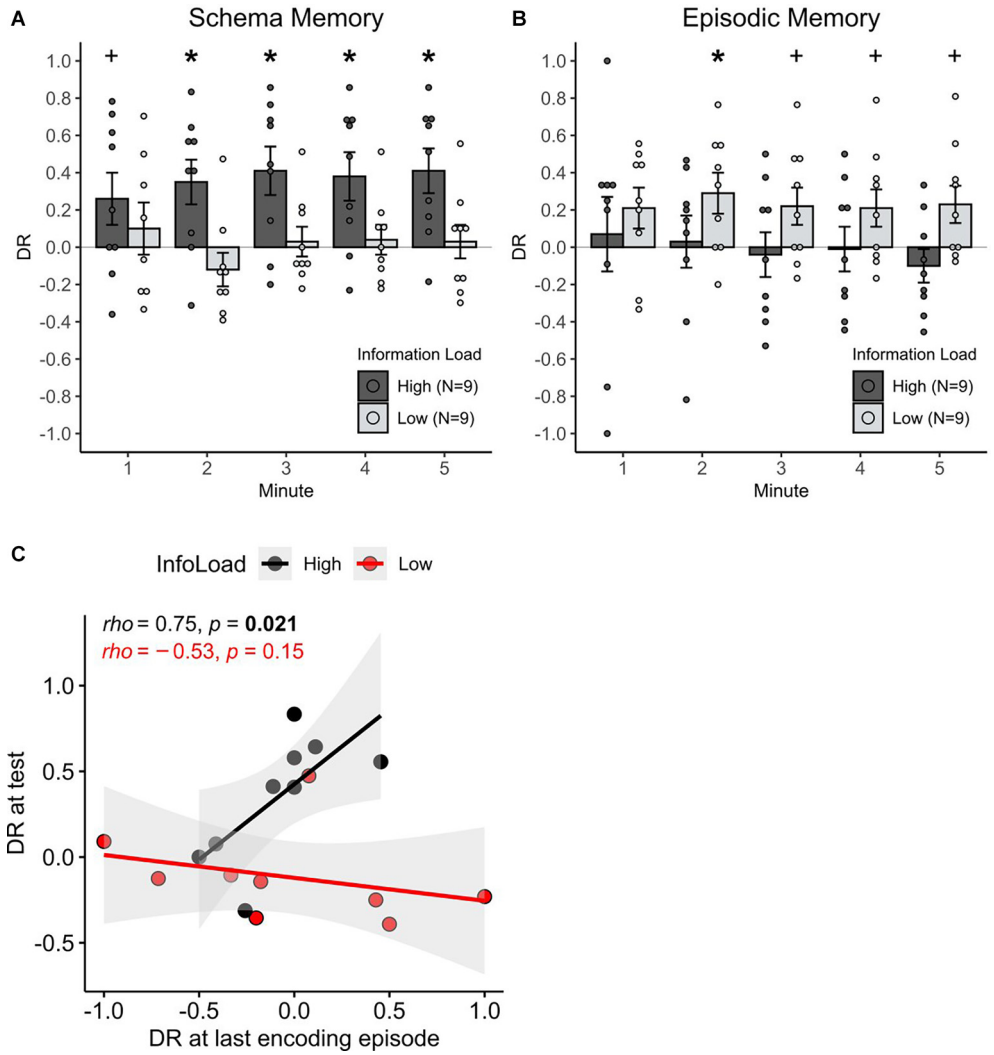

FIGURE 2 | The effect of information load on episodic and schema memory formation. **(A)** Mean + S.E.M. cumulative discrimination ratios during the 5-min test phase for the schema version of the task (n = 18), and **(B)** for the episodic version of the task (n = 18). Red asterisks indicate significant (above chance) memory per min ($^*p < 0.05$, $^+p < 0.1$). Schema memory for the spatial rule was expressed only in the high, but not in the low information load condition. Episodic memory was transiently expressed only in the low information load condition (2nd min). Significance for the information load (high, low) × memory type (schema, episodic) interaction ($p < 0.001$) points to an effect of episodic vs. schema memory in opposite directions. **(C)** Correlations of the discriminatory exploration towards the partially occupied location (DR > 0) at the last encoding episode in the high and low information load condition of the schema version of the task and the respective memory performance at the test. Ratios are taken from the first 2 min of the encoding and test episodes since memory expression was clearly present in that minute. Correlations significantly differ between the groups ($p < 0.01$). Note the significant positive correlation for the high information load condition suggests an emergent schema representation during the last encoding episode is predictive for schema memory recall at the test.

Whether these two processes can occur in parallel or compete with each other, is unknown. On the one hand, previous studies have demonstrated that the consolidation of declarative memories is impaired if the information load during learning is too large (Feld et al., 2016; Kolibius et al., 2021). On the other hand, it is known that schema learning benefits from higher loads of information, from which gist information can be extracted (Tse et al., 2007; Wang and Morris, 2010). Against this backdrop, the results of this study suggest that the formation of detailed episodic memory and generalized schema memory for a rule, indeed, depends on information load during learning in opposite direction. Animals were able to recognize a violation of the spatial rule present during encoding 24 h earlier only when exposed to eight episodes, but not to just four episodes during encoding. In contrast, episodic details of the last encoding episode were only retained after four but not eight consecutive encoding episodes.

The effect of information load on the formation of declarative memory has been previously tested in humans using a word-pair retention task (Feld et al., 2016; Kolibius et al., 2021). Learning large lists of word pairs, i.e., a high information load during learning, did not benefit from sleep-dependent memory consolidation processes, in contrast to learning shorter word

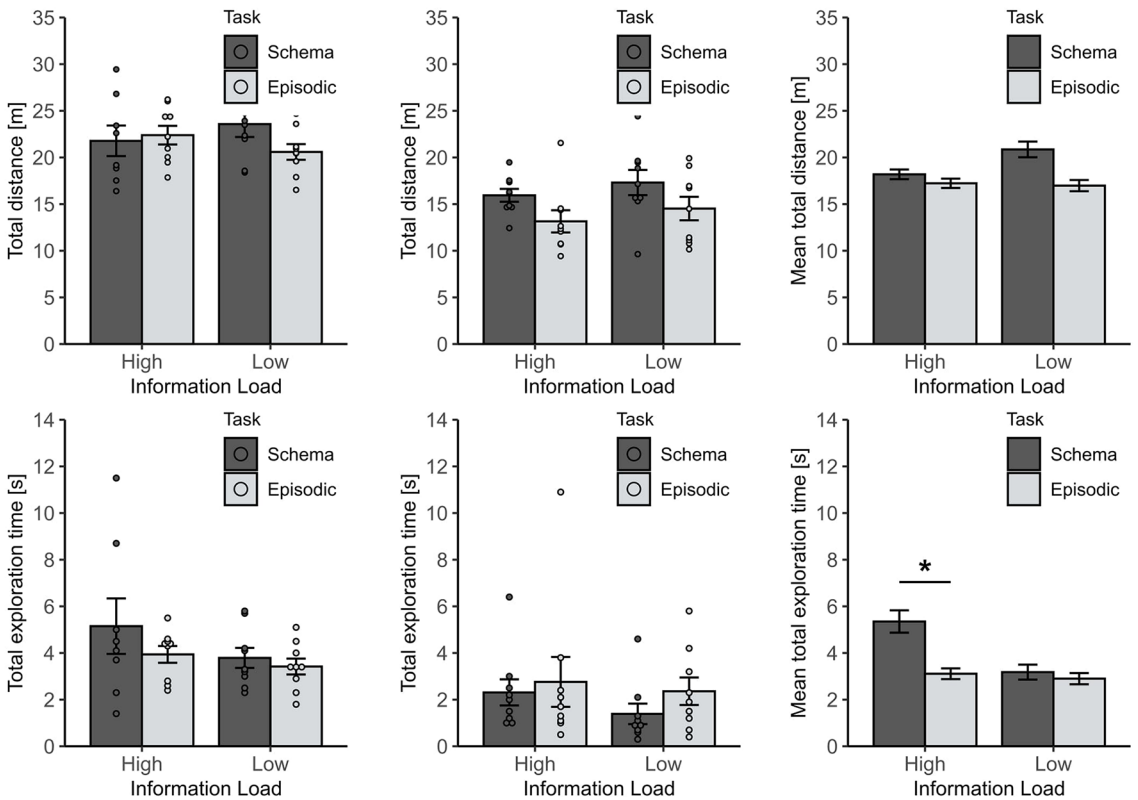

FIGURE 3 | Locomotion parameters for encoding episodes to assess unspecific motivational differences between experimental groups. **(A)** For the first encoding episode traveled distance and total exploration time were comparable across conditions (all $p > 0.1$). **(B)** For the last encoding episode (either the fourth or eighth in the low or high information load condition, respectively) the traveled distance decreased more in the high than in low-information load condition ($p = 0.019$), whereas the total exploration time remained comparable across groups ($p > 0.3$). **(C)** Mean distance traveled over all encoding episodes was lower in the high than in the low information load condition ($p = 0.018$). Unexpectedly, the mean total exploration time across all episodes depended on an interaction of Information Load and Type of Memory ($p = 0.037$) with this effect largely driven by higher mean exploration time in the High Load/Schema condition (lower panel). Statistically controlling for this effect did not change results for retrieval (see text). *$p < 0.05$.

pair lists. To explain this difference, it was proposed that active systems memory consolidation, a process that likely operates during sleep, is capacity-limited (Kolibius et al., 2021). We did not systematically assess to what extent our rats slept after the encoding phase, however, all experiments were carried out in the morning between 8:00 a.m. and 2:00 p.m. when sleep pressure in rodents is high (Van Twyver, 1969). Hence, post-encoding sleep might have been a factor significantly contributing to the present results in which the rats expressed episodic memory, although only transiently, in the low but not in the high information condition. Alternatively, decreased episodic memory formation with high loads of information encoded may be viewed as a consequence of increased non-specific interference, independent of the occurrence of sleep after encoding (Wixted, 2004; Yonelinas et al., 2019). Indeed, it has been suggested that familiarity-based memories are especially sensitive to interference (Sadeh et al., 2016), which is of importance as the memory test in the object-location preference task relies on familiarity. We aimed to reduce the interference in the task by testing episodic memory for the last encoding episode. However, while this strategy is sufficient for reducing retroactive interference, it cannot rule out proactive interference, i.e., the process in which previously learned information impairs the learning of new information (Brawn et al., 2018). In this view, the effect of information load on episodic memory might be mediated by proactive interference created by the task.

The opposite dependencies of schema vs. episodic memory performance on information load during encoding in our study hints toward a competitive relationship between the formation of these types of memory. A factor that could explain this competitive relationship might be the generally limited capacity of hippocampal networks for processing episodic memory information. In adapting the OPR task to the purpose of the present study, each encoding episode of the task used a unique object-location pairing. Accordingly, in the episodic version of the task, the information load scaled linearly with each additional encoding episode. It is likely that, with an increasing number of unique episodes, the capacity for episodic memory processing

in hippocampal networks is at some point surpassed, and the information is forgotten, be it during sleep to prevent episodic memories from undergoing active systems consolidation, or during wake to mutually weaken episodic memories through interference. As to the episodic memory test, this scenario would explain the absence of episodic memory in the high information load, but it might also partly account for the low information load condition, where the expressed episodic memory for the last encoding episode was rather weak and only transient (i.e., in the 2nd minute). Conversely, in the schema version of the task each encoding episode adds information about the spatial rule and information density for the rule, therefore, decreases over an increasing number of encoding episodes. At the level of hippocampal networks, this decrease in information density could be associated with an increased representational overlap between the individual encoding episodes that eventually facilitates abstraction of a more general schema memory for the spatial rule (Lewis and Durrant, 2011). If so, the postulated capacity limit should not interfere with the test of memory for the rule. Indeed, our data together with the inferred increase in representational overlap possibly facilitating schema memory formation, is also well in line with findings indicating that more repetitions are beneficial for the formation of schema memory, whereas a low information load at learning might not be sufficient (Tse et al., 2007; McKenzie et al., 2014).

The conclusion that the opposite effects of information load on schema vs. episodic memory reflect a competitive relationship between these memories, may be questioned based on the fact that, rather than probing both kinds of memory with the same stimulus materials, task stimuli differed between the schema and episodic versions of the task, with only the former comprising the spatial rule across encoding episodes. However, using the same behavioral readout (i.e., exploration of novelty) for probing episodic and schema memory in the used adaptation of the OPR-based task, it is basically impossible to independently assess both kinds of memory on an identical set of stimuli during encoding, simply because a set of encoding episodes that allows for abstracting a spatial rule across episodes, necessarily allows for the simultaneous formation of episodic memories for the individual encoding episodes. Specifically, this means that the schema version of the task cannot be used to independently assess episodic memory. In principle, an assessment of memory for an individual encoding episode would require that at the test, only one of the objects of the respective episode is displaced such that the original spatial configuration of this episode changes but the rule across episodes is continued (i.e., one object stays at the always occupied location and the other switches to the formerly not occupied partially occupied location). Such test configuration, however, does not allow for a valid test of pure episodic memory, as it could well be biased by the continuation of the rule and the resulting rule knowledge (making the respective episodic change in the location of the object appearing less novel). Moreover, animals forming memory in a cumulative manner across multiple episodes have been found to prefer exploring the less often occupied location over the always occupied location, independently of whether or not the less often occupied location violates an emergent rule (Genzel et al., 2019). Note, for testing schema memory separately from episodic memory, we, therefore, displaced both objects of the respective episode to another location (one violating the rule and the other deviating from the spatial configuration of this particular episode). This test configuration is expected to elicit parallel exploration driven by episodic memory and exploration driven by schema memory, but only an activated schema memory would drive a differential exploration towards the object in the novel location, i.e., the one violating the spatial rule, as it was found in the high information condition.

However, despite the proposed competitive relationship between episodic and schema memory, based on the present findings, it is impossible to rule out alternative explanations. Since episodic memory could not be assessed in the schema version of the task, one might alternatively explain the effect of information load on schema memory based on the occurrence of retro- and proactive interference across episodes (Wixted, 2004). For instance, if animals in the low load condition of the schema memory task had formed, at the test phase, both schema memory as well as episodic memory for the first encoding episode, both objects on the never-occupied (rule-violating) location and on the partially occupied (rule-continuing) location, would represent "unfamiliar" locations, and the zero-discrimination found in this condition would not indicate the absence of schema memory, but the sole presence of episodic memory or the joint presence of episodic and schema memory that cancel each other out. In this scenario, an increase of interference as a result of higher information load results in a weaker episodic memory and, thus, a clearer schema memory expression. In order to test whether or not schema memory is actually formed already after four encoding episodes (i.e., in the low load condition), our schema memory task that was based on a classical OPR-task design would need further modification. For example, a third object could be added to each episode such that there is repeating (i.e., schema relevant) information and unique (i.e., episodic) information available that can be contrasted in a memory test phase. Such modification clearly separating shared and item-unique information would make the task similar to the Satellite task used in humans (Schapiro et al., 2017). However, it still would not solve the problem of interpreting a zero-discrimination ratio indicating either the absence of memory or the presence of both episodic and schema memory that cancel each other out, rendering appropriate control conditions vital to the interpretation of behavioral effects.

To explain our behavioral results, one might also refer to the concept of habituation, i.e., after eight encoding episodes, rats at the test in the schema version of the task preferentially explored the object that violated the rule because they were more habituated to the presence of an object in the partially occupied locations. However, the process of habituation when considered as a learning process across episodes with differing stimulus configurations does not exclude processes of schema memory formation, but would rather explain the changes in behavior at a different epistemological level. Note, that the objects used in the different episodes were clearly discriminable for the rats and, interestingly, a supplementary analysis revealed no clear signs of habituation across encoding episodes, in terms of a decreased

exploration toward the always occupied location across episodes (see **Supplementary Figure 3** for respective learning curves).

The formation of schema memory is often thought to be a slow process that evolves during systems consolidations over longer time intervals, i.e., days and even weeks (Walker and Stickgold, 2010; Lewis and Durrant, 2011; Dudai et al., 2015), although there is no minimum time required to form a schema. In this study schema memory for a spatial rule was formed in ∼3 h and retrieved within 24 h in the high information load condition. Does this quick timescale contradict the concept of schema memory? Previous studies indicate that the speed at which schema memories are formed essentially depends on the presence of pre-existing knowledge into which respective information can be readily integrated (Gilboa and Marlatte, 2017). Even schema memories that derive from reoccurring patterns over multiple episodes may rather rapidly form when the relevant information can be readily assimilated into pre-existing representations (Tse et al., 2007). Pre-existing knowledge may have also accelerated schema memory formation in the present experiment: The rats were thoroughly habituated to the arena before the experiments to develop an allocentric spatial map of the arena environment. Also, experiences of the general procedures including the habituation to the experimenter, the rat's journey back and forth to the experimental room, etc. might have formed memories representing an abstract knowledge about the commonalities across days. These and related representations might have served as a scaffold facilitating the formation of schema memory arising in the same environmental context, especially under high information load conditions. An interesting question not addressed here is whether in low information load conditions the abstraction of a schema memory for the spatial rule would unfold with longer periods of active consolidation (Nader et al., 2000; Binder et al., 2012; Dudai, 2012).

Overall, the present results indicate that the amount of encoded information impacts, in opposite directions, the formation of schema and episodic memory. A factor contributing to this effect might be the limited capacity of hippocampal networks for processing memory information, enforcing representational overlap to augment schema formation in conditions of high information load, whereas episodic memory can freely form in conditions of low information load. However, modifications to the task design are needed to directly assess the proposed competitive relationship. Our study demonstrates in principle that OPR-based tasks offer a promising approach to the combined study of episodic and schema memory dynamics in rodents.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Baden-Wuerttemberg state authorities.

AUTHOR CONTRIBUTIONS

MC and MH carried out the experiments. MH performed all the analyses. MC, MI, and JB conceived the original experiment. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.923713/full#supplementary-material.

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Dopamine activity on the perceptual salience for recognition memory

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To survive, animals must recognize relevant stimuli and distinguish them from inconspicuous information. Usually, the properties of the stimuli, such as intensity, duration, frequency, and novelty, among others, determine the salience of the stimulus. However, previously learned experiences also facilitate the perception and processing of information to establish their salience. Here, we propose "perceptual salience" to define how memory mediates the integration of inconspicuous stimuli into a relevant memory trace without apparently altering the recognition of the physical attributes or valence, enabling the detection of stimuli changes in future encounters. The sense of familiarity is essential for successful recognition memory; in general, familiarization allows the transition of labeling a stimulus from the novel (salient) to the familiar (non-salient). The novel object recognition (NOR) and object location recognition (OLRM) memory paradigms represent experimental models of recognition memory that allow us to study the neurobiological mechanisms involved in episodic memory. The catecholaminergic system has been of vital interest due to its role in several aspects of recognition memory. This review will discuss the evidence that indicates changes in dopaminergic activity during exposure to novel objects or places, promoting the consolidation and persistence of memory. We will discuss the relationship between dopaminergic activity and perceptual salience of stimuli enabling learning and consolidation processes necessary for the novel-familiar transition. Finally, we will describe the effect of dopaminergic deregulation observed in some pathologies and its impact on recognition memory.

KEYWORDS:

novelty, catecholamines, saliency, contextual, perception

Introduction

Organisms are continuously exposed to several stimuli and events in their environment across their lifespans. Nevertheless, individuals must efficiently and effectively guide their behavior according to the perceived relevant stimuli. The continuous processing of incoming information demands considerable cognitive effort. Therefore, selecting, filtering, and processing information is essential to preserve proper cognitive function. In this regard, the relevant information is processed with less cognitive interference, characterized by the competition of information in eliciting cognitive processes

(Grachev et al., 2001) compared to neutral or inconspicuous stimuli. Selecting relevant information from the environment is easily achieved when these stimuli are intrinsically salient due to their physical properties. Thus, salience refers to the phenomenon by which a stimulus highlights or is set apart from the environment (Uddin, 2015). Generally, a salient stimulus attracts attentional resources bottom-up to facilitate information processing (Santangelo, 2015), where the stimulation drives cognitive processes that contrasts with the surroundings. Bottom-up processing states that the stimuli's physical attributes originate from sensory information facilitating salience and perception (Riener, 2019). In this regard, perceptual processing determines the attention and cognition required for a proper behavioral reaction to the stimuli in the environment (Goldstein and Cacciamani, 2021). However, there is evidence suggesting that the physical properties of the stimuli are not the only factors that drive information processing. Top-down processing, also called knowledge-based processing, refers to the "internal" factors of the observer acquired by previous experiences (Awh et al., 2012). Top-down processing is driven by cognition, starting with memory and expectations that affect salience and perception (Riener, 2019). Therefore, the brain hierarchizes salient information according to the physical stimuli properties and the organism's experience, facilitating perception of the stimuli.

Current evidence indicates that perception and memory interact to direct and control driving attentional and cognitive processes actions (for a review, Heurley and Ferrier, 2015). A salient stimulus is more prone to be integrated into memory traces than non-salient stimuli. Even though many stimuli appear to be non-salient due to their intrinsic low-intensity properties; they can become salient based on their meaning, consequences, or relationship with other stimuli in the environment (Santangelo, 2015). Therefore, previous experiences modulate stimuli recognition by comparing them with stored information and adjusting salience processing (Riener, 2019). Here, we propose the term perceptual salience referring to how memory modulates the integration of inconspicuous stimuli into a relevant memory without enhancing the initial sensory perception.

Memory is a fundamental adaptative mechanism that promotes organisms' survival by identifying relevant environmental changes. Across their lifespan, animals experience several episodes associated with important information about food, shelter, and danger, among others. Thus, individuals need to recall those specific events and appropriately modify their behavior to survive. The encoding, integration, and retrieval of experienced information is defined as memory (Squire, 2009). Overall, memory formation requires acquiring information by learning events during exposure to stimuli. Then, memories are integrated into long-lasting traces through chemical and structural modification *via* protein synthesis (McGaugh, 2000; Bisaz et al., 2014). When necessary, internal and external cues promote selection, reactivation, and assessment of information, modulating the behavioral outcomes, a process called memory retrieval (Ben-Yakov et al., 2015; Frankland et al., 2019). During memory retrieval, memories undergo a consolidation-like process called reconsolidation, where memory updating may occur (Nader et al., 2000; Sara, 2000; Lee et al., 2017; Rodriguez-Ortiz and Bermúdez-Rattoni, 2017). Memory engages distinct neural circuits or systems accordingly to the type of information, inducing cellular and molecular changes that support memory maintenance (Nadel and Hardt, 2011).

According to the awareness during retrieval, memory systems have been classified into declarative and non-declarative. Non-declarative memories are characterized by integrating information associated with habits, motor learning, and associative learning. The most common example is the information accessed without conscious recall. Conversely, declarative memories are recalled consciously and are related to facts (semantic memory) and events (episodic memory; Squire, 2009; Nadel and Hardt, 2011). Specifically, episodic memory integrates "where," "what," and "when" an event happened into a spatiotemporal context (Tulving, 2002). Therefore, an essential aspect of episodic memory is the judgment of whether a recent experience, including subject, location, or event, has been previously experienced or encountered. Episodic memory integrates information related to environmental changes, facilitating the identification of different information modalities, including faces, places, sounds, objects, or changes in the context. The integrated information allows the discrimination of novel events from familiar ones. Thus, recognition memory involves familiarization by acquiring, consolidating, retrieving, and updating experienced events in a space-time frame (Squire and Zola, 1996; Tulving, 2002; Balderas et al., 2015; Morici et al., 2015).

In general, recognition memory incorporates two differential processes: Recollection and Familiarity (Brown and Aggleton, 2001; Merkow et al., 2015). Familiarity is the ability to judge whether a particular stimulus or event has already been experienced (Mandler, 1980). In contrast, recollection retrieves the stimuli or events' characteristics (qualitative dimension; Evans and Wilding, 2012). Thus, exposure to novelty (salient stimulus) triggers a maximum behavioral response that is progressively reduced during subsequent presentations (familiar, non-salient stimulus). The novelty transitions to familiarity are gradual shifts caused by learning (Henson and Gagnepain, 2010) and neuronal plasticity changes (Lisman et al., 2011). Recollection of contextual events and their behavioral responses occur by activating several brain regions (Kafkas and Montaldi, 2014), like the entorhinal cortex (Knierim, 2015), hippocampus (Barker and Warburton, 2011), and the prefrontal cortex (Akirav and Maroun, 2006). In comparison, the items' familiarity variations rely on parahippocampal (perirhinal, entorhinal, and postrhinal; Brown and Aggleton, 2001;

Yonelinas, 2002; Evans and Wilding, 2012; Merkow et al., 2015) and insular (Bermudez-Rattoni et al., 2005; Balderas et al., 2008) cortices of the brain. Within these structures, changes in the neurotransmitters involved in the transition from novelty to familiarity include elevation in acetylcholine, noradrenaline, and dopamine which gradually diminish after the consecutive exposure to the stimulus (Miranda et al., 2000; Osorio-Gómez et al., 2016, 2017; Rodríguez-García and Miranda, 2016). The activation of the same neurotransmission systems occurs in different areas of the brain depending on memory recollection of events or stimuli recognition. Even though many neurotransmitters are involved in recognition memory, the catecholaminergic system is of particular interest due to its modulatory effect on synaptic plasticity and memory processes that might impact perceptual salience (Jay, 2003; Lisman et al., 2011; Takeuchi et al., 2016; Yang et al., 2017).

Finally, we will review how cognitive impairments are directly related to dopaminergic dysfunctions, impacting recognition memory in pathologies such as Alzheimer's disease (Guzmán-Ramos et al., 2012; Moreno-Castilla et al., 2017), schizophrenia (Brisch et al., 2014), and Parkinson's disease (Aarsland, 2016). Abnormal functioning in several brain regions has been related to recognition memory detriments observed during spontaneous exploration tasks in the animal model used to study these processes.

Spontaneous object exploration

Spontaneous novel object recognition tasks are widely used to assess long-term recognition memory's neurobiological mechanisms. Novel object recognition (NOR) and object location recognition memory (OLRM) are the most common behavioral paradigms employed to determine the processes of acquisition, consolidation, retrieval, and updating of recognition memory (see **Figure 1**). NOR and OLRM are simple tasks based on the rodents' innate preference to explore novel stimuli than familiar ones (Ennaceur and Delacour, 1988; Chan et al., 2018). Both paradigms consist of at least three sessions: a handling and habituation period to an empty open field. Then, a sample phase (acquisition session), where animals explore novel objects for first-time. Finally, a test session (retrieval) where animals discriminate and identify a novel object in the case of NOR or the displaced one in the case of OLRM (Ameen-Ali et al., 2015; Chan et al., 2018). In addition, novel information can be integrated during retrieval and updating recognition memory (Balderas et al., 2015; Kwapis et al., 2020; Wright et al., 2020; see **Figure 1**). These tasks allow us to assess the ability of animals to recognize environmental changes caused by exposure to a novel object or a novel spatial configuration and help determine novel/familiar discrimination and recollection processes.

Several experimental approaches have evaluated the differential participation of several brain structures in the consolidation of recognition memory. Regarding OLRM, the hippocampus is a crucial brain structure in spatial-dependent tasks. Hippocampal lesions impair OLRM (Save et al., 1992; Mumby et al., 2002; Barker and Warburton, 2011). Particularly, CA3 lesions (Lee et al., 2005; Hunsaker et al., 2008) or its pharmacological inactivation (Barbosa et al., 2012) hinder spatial novelty discrimination. In addition, pharmacological inactivation of the CA1 portion impairs OLRM (Assini et al., 2009). Thus, the dorsal CA1 and CA3 portions are related to acquiring, consolidating, and retrieving contextual information (Brown and Aggleton, 2001; Barker and Warburton, 2011; Moreno-Castilla et al., 2017). Concerning NOR, this task depends on the insular cortex (Bermudez-Rattoni et al., 2005; Balderas et al., 2008), the perirhinal cortex (Warburton et al., 2003; Winters et al., 2004; Winters and Bussey, 2005; Balderas et al., 2013b), and the ventromedial prefrontal cortex (Akirav and Maroun, 2006). However, the involvement of the hippocampus in NOR has been controversial (Mumby, 2001; Balderas et al., 2008; Barker and Warburton, 2011; Haettig et al., 2011). Evidence suggests that the functional integrity of the hippocampal activity is required in NOR consolidation (Rossato et al., 2007; Myskiw et al., 2008; Cohen et al., 2013; Furini et al., 2014). For many years it was assumed that hippocampal activity was necessary only when recalling objects in a particular context (Barker and Warburton, 2011). Anisomycin administration into the dorsal hippocampus immediately after the sample phase impairs long-term but not short-term object-in-context recognition memory (Balderas et al., 2008). The object-in-context task is a spontaneous exploration paradigm in which animals spend more time exploring familiar objects within a novel context (salient information) than in a familiar one (non-salient information). However, conflicting results might be explained by differences in experimental approaches (lesions vs. temporal inactivation). The use of a particular behavioral protocol and not merely by the type of information (what vs. where; for review, please see Cohen and Stackman, 2015).

Catecholaminergic system involvement in NOR and OLMR

The catecholaminergic system is strongly related to cognitive processes and plays a vital role in the modulation of recognition memory (Yang et al., 2017; Titulaer et al., 2021). Exposure to novel stimuli or contextual information induces an elevation in catecholamines and disruption in catecholaminergic activity hinders recognition memory (Guzmán-Ramos et al., 2012; Moreno-Castilla et al., 2017). Dopaminergic and noradrenergic systems regulate neuronal plasticity events related to memory formation and consolidation. So, any alteration in the catecholaminergic system elevates the probability of cognitive impairments, including recognition

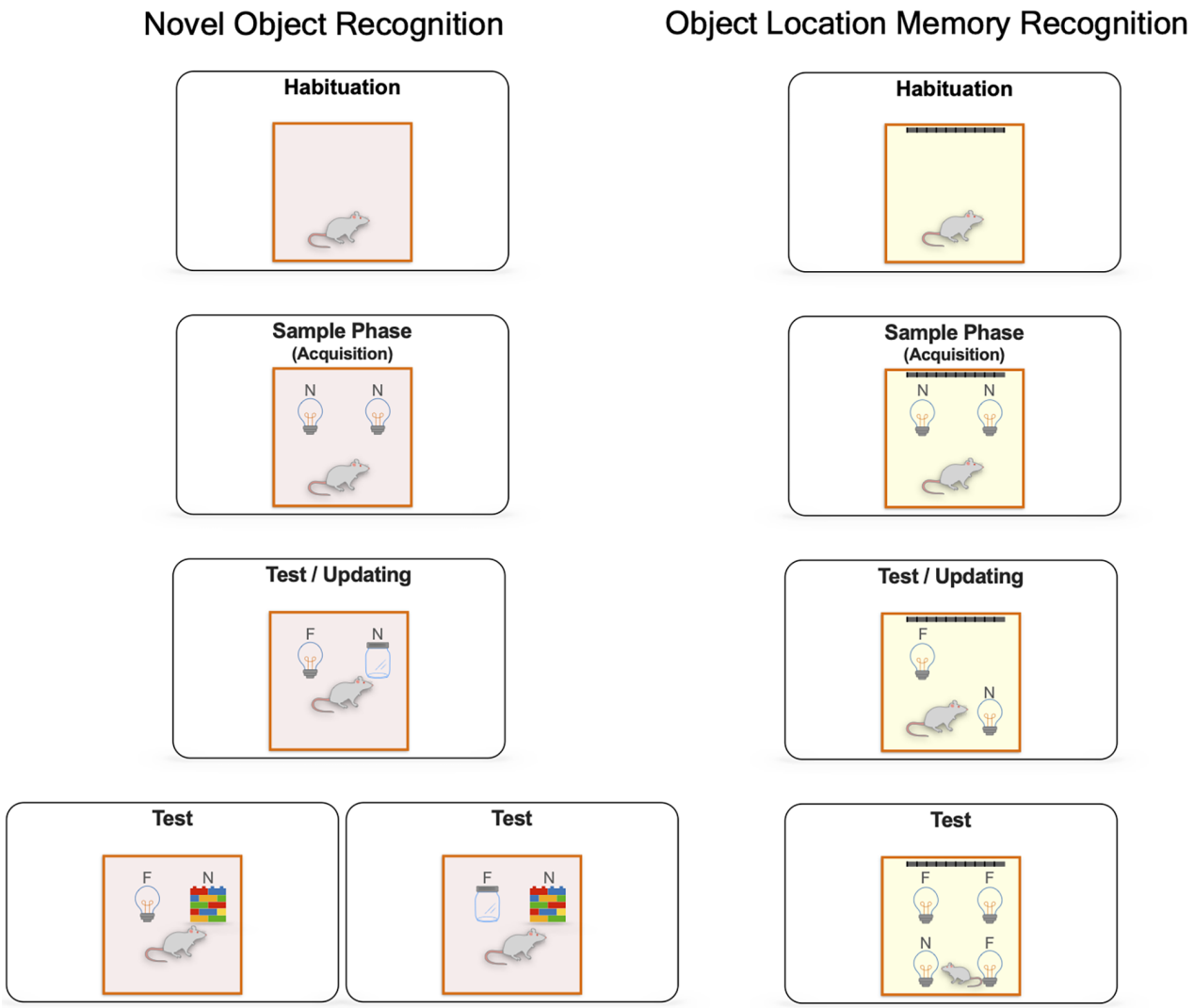

FIGURE 1
General procedures for spontaneous object exploration. Novel object recognition (NOR) and object location recognition memory (OLRM) paradigms include different sessions: a handling and habituation period to an empty open field to reduce stress and promote the exploratory activity; a sample phase, where the animal is given a period to freely explore two identical objects. During the test session, short-term or long-term assessment of recognition memory is determined by the length of the retention interval, in this session the animal freely explores a different novel object (jar) in the case of NOR or the displacement of an object to a novel location in the case of OLRM. Finally, recognition memory updating is evaluated if a new third object is presented (wall of brick toys) in a NOR task or when a new displaced position is presented in an OLRM task. In general, animals that remember the familiar object of the familiar spatial configuration will spend more time exploring the novel object or the novel spatial configuration Novel (N) and Familiar (F).

memory. The main catecholaminergic inputs arise from the locus coeruleus (LC) and the ventral tegmental area (VTA), innervating several brain structures through the mesolimbic and mesocortical pathways (Ungless, 2004; Bromberg-Martin et al., 2010; Takeuchi et al., 2016).

Regarding norepinephrine, the LC supplies projections into several parts of the brain, including the medial temporal lobe (Pudovkina et al., 2001; Aston-Jones and Cohen, 2005; Morilak et al., 2005; Kempadoo et al., 2016). The noradrenergic system plays a critical role in memory consolidation processes by modulating plasticity-related events (McGaugh, 2000, 2013, 2015; Barsegyan et al., 2014). Norepinephrine activates α (α1 and α2) and β (β1, β2, and β3) receptors. Stimulation of α1 receptors increases Ca^{2+} and diacylglycerol intracellular levels, promoting the activation of phospholipase C and the subsequent activation of protein kinase C; this signaling pathway modulates neuronal changes necessary for memory establishment (Perez, 2020). Furthermore, the activation of β receptors promotes adenylate cyclase activity that increases cAMP levels. This augmentation results in the activation of protein kinase A, which ultimately promotes gene expression and memory consolidation (O'Dell et al., 2015).

LC activity increases its firing response after presenting novel objects or contexts (Sara et al., 1994; Vankov et al., 1995; Pudovkina et al., 2001; Wagatsuma et al., 2018). Recent evidence indicates that the LC projects dopaminergic and noradrenergic terminals into the dorsal hippocampus (Kempadoo et al., 2016), enhancing spatial memory consolidation through D1-like receptor activity (Takeuchi et al., 2016). Moreover, the catecholaminergic denervation through the administration of 6-OHDA into the hippocampus interferes with OLRM formation (Moreno-Castilla et al., 2017). A similar lesion in the shell subregion of the nucleus accumbens impairs object location memory (Nelson et al., 2010). Pharmacological activation of the noradrenergic receptors through the systemic administration of epinephrine improves recognition and memory consolidation (Dornelles et al., 2007). Similarly, activation of β receptors within the basolateral amygdala enhances object-in-context recognition memory (Barsegyan et al., 2014), NOR (Chen et al., 2022), and OLRM consolidation (Roozendaal et al., 2007; Song et al., 2021). The noradrenergic system presumably promotes memory consolidation due to arousal effects; since the basolateral amygdala modulates the dorsal hippocampus and insular and prelimbic cortices through the noradrenergic system (Barsegyan et al., 2019; Chen et al., 2022). Likewise, norepinephrine administration into the hippocampus immediately after the sample session promotes NOR persistence (Mello-Carpes et al., 2016). Thus, exposure to novel objects or contextual information enhances memory consolidation through LC modulation, noradrenaline release within the amygdala, and the corelease of norepinephrine and dopamine in the hippocampus. In addition, changes in norepinephrine levels would be associated with relevant stimuli detection, regulating arousal, improving cortical function, and enhancing subsequent cognitive functions such as attention and motivation (Aston-Jones and Cohen, 2005).

The other catecholaminergic neurotransmitter is dopamine, produced in midbrain neurons within the substantia nigra and the VTA (Baik, 2020). Dopamine release from the substantia nigra is mainly involved in controlling motor function and goal-directed behaviors (Grillner et al., 2008). However, dopamine release from the VTA and the LC terminals into the nucleus accumbens, prefrontal cortex, and medial temporal lobe (hippocampus, perirhinal, insular, parahippocampal, and entorhinal cortices) is involved in the formation and maintenance of declarative memories such as recognition memory (de Lima et al., 2011; Kempadoo et al., 2016; Takeuchi et al., 2016; Moreno-Castilla et al., 2017). In general, dopamine is a neuromodulator that modifies functional connectivity during synaptic plasticity (Jay, 2003; Lisman et al., 2011; Otani et al., 2015; Yang et al., 2017). These modifications occur after the activation of metabotropic receptors and the later induction of signaling pathways cascades, promoting the enhancement of neuronal plasticity. Mainly, dopamine activates D1-like (D1 and D5) and D2-like (D2, D3, and D4) receptors; activation of D1 receptors triggers Gs protein inducing an augmentation of cAMP and the subsequent activation of protein kinase A (Undieh, 2010), regulating conductance of NMDA receptors *via* phosphorylation of NR1 and NR2 subunits (Chen et al., 2004; Murphy et al., 2014). Moreover, activation of D1 receptors promotes AMPA (Mangiavacchi and Wolf, 2004; Rozas et al., 2015) and NMDA (Gao and Wolf, 2008; Li et al., 2010) receptors externalization. In the case of D2-like receptors, their activation inhibits adenylate cyclase through Gi proteins, suppressing neurotransmitter release from terminals (Neve et al., 2004).

Dopamine plays an essential role in recognition memory since the dopaminergic neuronal activity is modified by novel and salient stimuli (Ljungberg et al., 1992; Ungless, 2004). The VTA is a dopaminergic nucleus that displays changes in electrical activity associated with the presentation of novel stimuli (Ljungberg et al., 1992; Schultz, 1998; Düzel et al., 2009), increasing dopaminergic levels within the nucleus accumbens (Legault and Wise, 2001; Leonibus et al., 2006), striatum (Ihalainen et al., 1999), the dorsal and ventral hippocampus (Ihalainen et al., 1999; Mello-Carpes et al., 2016; Moreno-Castilla et al., 2017; Hernández-Ramírez et al., 2021; Titulaer et al., 2021), the prefrontal cortex (Feenstra and Botterblom, 1996; Ihalainen et al., 1999; Feenstra et al., 2000), and the insular cortex (Guzmán-Ramos et al., 2010, 2012; Osorio-Gómez et al., 2021). Therefore, the dopaminergic system has been related to novelty detection that triggers recognition memory establishment (Rossato et al., 2013; Otani et al., 2015; Moreno-Castilla et al., 2016; Yang et al., 2017). Object and location recognition memory depends on catecholaminergic activity. Exploring novel objects induces dopamine release into the insular cortex, but CA1 hippocampal dopamine remains unaltered during the sample phase (Guzmán-Ramos et al., 2012). Significantly, hippocampal catecholaminergic denervation by 6-OHDA administration impedes OLRM but spares NOR (Moreno-Castilla et al., 2017), indicating that the hippocampal dopaminergic activity is not involved in NOR formation or retrieval. Hence, the fine modulation of the dopaminergic system is required to establish recognition memory appropriately.

The inactivation of VTA (Rossato et al., 2013) or the denervation of the mesolimbic-cortical dopaminergic terminals (Stephen Fink and Smith, 1980) hinders NOR consolidation. While an excess of dopaminergic levels, caused by knocking out the expression of the dopamine transporter, impedes NOR formation (Chang et al., 2020). Similarly, systemic administration of methamphetamine, an enhancer of catecholamines release (Belcher et al., 2008; Camarasa et al., 2010), or the blockade of D2 receptors or D4 receptors, impairs NOR establishment (Besheer et al., 1999; Woolley et al., 2003; Watson et al., 2012; Miyauchi et al., 2017). Moreover, the systemic activation of D1-like receptors hinders OLRM and NOR retrieval (Hotte et al., 2005; Pezze et al., 2015), while the inactivation of D3 (Watson et al., 2012) enhances novel recognition retrieval. Likewise, memory persistence

and memory retrieval in NOR are heightened after the systemic administration of a D1/D5 receptor agonist (Hotte et al., 2005, 2006; de Lima et al., 2011; see **Table 1**). Regarding the hippocampus, administering a D1 antagonist after the sample phase into the dentate gyrus impairs object recognition (Yang et al., 2017). However, administering a D1/D5 receptor antagonist into the dorsal hippocampus before or after the sample phase spares long-term NOR memory (Balderas et al., 2013a; Rossato et al., 2013). Conversely, the administration of a D1/D5 receptor antagonist into the perirhinal cortex before the sample phase spares short-term but prevents the consolidation of NOR (Balderas et al., 2013a). Moreover, the intracerebroventricular administration of D1-like receptors antagonist impairs spatial novel configuration learning (Lemon and Manahan-Vaughan, 2006), whereas blockade of D1 receptors within the prefrontal cortex or amygdala (Nagai et al., 2007; Rossato et al., 2013) impairs NOR consolidation (see **Table 2**).

All these results suggest that catecholaminergic activity, particularly dopaminergic modulation, is responsible for enhancing the consolidation of recognition memory. Exposure to novel stimuli induces dopamine release in several brain structures, facilitating memory establishment through the synthesis, tagging, and capture of proteins associated with synaptic plasticity generated by learning signals (Frey and Morris, 1998). In the absence of a neuromodulator, modified synapses return to the baseline level after learning, reducing the probability of consolidating memories (Takeuchi et al., 2016; Duszkiewicz et al., 2019). Therefore, it has been suggested that dopamine modulates learning signals that facilitate the consolidation of events (Montague et al., 2004) involved in the process of familiarity consolidation. Thus, dopamine might modulate perceptual salience consolidation signals, enabling recognition memory.

Dopamine and perceptual salience

As previously reviewed, dopaminergic signaling is widely associated with neuronal plasticity enhancement. In general, dopamine activates D1-like and D2-like receptors triggering a cascade of events that lead to cellular modifications and the induction of protein synthesis necessary for memory consolidation. Therefore, dopaminergic activity is related to the modulatory effect of object and location recognition memory establishment. Although dopaminergic activity contributes significantly to memory processes, it remains to elucidate the precise functional role of dopamine and its specific contribution to perceptual salience processing necessary for recognition memory evaluated through NOR and OLRM tasks. Novelty-related dopaminergic activity within several brain structures is involved in recognition memory. Dopamine release has been related to motivated behaviors and predicting and coding rewarding events (Schultz et al., 1993; Schultz, 1998). The evidence shows that VTA dopaminergic neurons increase their firing rate to signal reward (Berridge and Robinson, 1998; Nomoto et al., 2010; Fiorillo, 2013). Nevertheless, evidence exhibits that exposure to aversive stimuli also increases the electrical activity within VTA (Brischoux et al., 2009; Bromberg-Martin et al., 2010). Whereas unexpected stimuli prediction errors also modulate dopaminergic activity (Schultz, 1998). Therefore, novelty, the intrinsic value of the stimulus (valence), and unforeseen modifications in predicted events induce changes in dopaminergic response, probably on behalf of salience (Horvitz, 2000).

Consequently, exposure to novel stimuli triggers dopamine release facilitating memory consolidation (Balderas et al., 2013a; Osorio-Gómez et al., 2021). Notably, salient visual stimulation induces a short-latency electrical response in the substantia nigra (Comoli et al., 2003). This phasic dopaminergic activity is strongly related to salience (Bromberg-Martin et al., 2010; Barto et al., 2013; Cho et al., 2017). Intrinsically salient stimuli compete for attention, in which new and relevant visual stimuli drive attentional processes (Yantis and Hillstrom, 1994). Novelty-induced salience influences memories, attention, and motivation through dopaminergic activity (Puglisi-Allegra and Ventura, 2012). Salient stimuli prioritize the consolidation of the relevant over neutral information (Alger et al., 2019). Interestingly, it has been suggested that the brain is organized to promote the interaction of functional networks, including the salience network (Tsai et al., 2020). Evidence indicates that the insular cortex is a crucial node of the salience network (Uddin, 2015), integrating exteroceptive and interoceptive information (Seeley et al., 2007). The insular cortex might be involved in salience because of the multiple inputs arising from the amygdala, VTA, the dorsomedial nucleus of the thalamus, and the prefrontal cortex (Bermudez-Rattoni, 2014; Uddin, 2015; Gil-Lievana et al., 2020, 2022; Chen et al., 2022).

As mentioned, the physical properties of the stimulus drive cognitive processes contrasting it with the surroundings and attracting attentional resources facilitating information processing. However, the physical properties of the stimuli are not the only components that can direct information processing. Previous experiences modulate top-down processing, by which "internal" factors and cognition influence perception (Awh et al., 2012). Thus, information is hierarchized within the brain according to the physical properties of the stimulus or to the previously learned information related to that stimulus, facilitating the perception of the stimuli in future events. Consequently, perceptual salience requires integrating information into meaningful-related memories without changing the initial detection of the stimuli (see **Figure 2**).

The hippocampus is involved in detecting salient spatial stimuli; hippocampal formation increases its activity when new contextual information is presented. This novelty signal is transferred to the VTA and contributes to the activation

TABLE 1 Systemic pharmacological effects of catecholaminergic drugs on NOR and OLRM performance.

	Drug	Mechanism	Time of administration	Task	Effect on memory	References
α and β						
	Epinephrine	Agonist	Post-training	NOR	↑	Dornelles et al. (2007)
α2						
	Yohimbine	Agonist	Post-training	NOR	↑	Roozendaal et al. (2007) and Song et al. (2021)
	Yohimbine	Agonist	Post-training	OLRM	↑	Song et al. (2021)
Dopamine uptake transporter						
	Methamphetamine	Stimulates release of dopamine	Chronic, one week before training	NOR	↓	Belcher et al. (2008) and Camarasa et al. (2010)
D2/D3						
	Eticlopride / Raclopride	Antagonist	Before retrieval/Before training	NOR	↓	Besheer et al. (1999) and Woolley et al. (2003)
D1/D5						
	SCH-23390	Antagonist	Before retrieval	NOR	↓	Besheer et al. (1999)
	SKF81297	Agonist	Before retrieval	OLRM	↓	Hotte et al. (2005)
	SKF81297	Agonist	Before retrieval	NOR	↓	Hotte et al. (2005)
	SKF38393 / SKF81297	Agonist	Post- training/Before retrieval	NOR	↑	Hotte et al. (2005, 2006) and de Lima et al. (2011)
D2						
	PD128, 907	Antagonist	Before retrieval	NOR	↓	Watson et al. (2012)
D3						
	S33084	Antagonist	Before retrieval	NOR	↑	Watson et al. (2012)
D4						
	L-745, 870	Antagonist	Before training	NOR	↓	Miyauchi et al. (2017)

↑ indicates enhanced memory and ↓ indicates impaired memory.

pattern observed in the VTA during novelty seeking (Lisman and Grace, 2005). For instance, the temporal inactivation of the VTA impairs NOR consolidation (Rossato et al., 2013), indicating that the dopaminergic system is essential for object recognition consolidation. This information suggests that the hippocampus and the dopaminergic inputs from the VTA and the LC form a functional loop that detects novelty and compares this information to previously integrated memories. Thus, dopaminergic activity might control the entry of crucial adaptative information and promotes subsequent integration into long-term memory through modification of synaptic plasticity (Lisman and Grace, 2005; Lisman et al., 2011).

There are illustrative examples of perceptual salience modulation *via* dopaminergic regulation. Optogenetic stimulation of the dopaminergic neurons within the VTA enhances behavioral response (taste neophobia) to the low-intensity stimulus facilitating taste recognition memory consolidation (Gil-Lievana et al., 2022). This effect was probably to bottom-up salience enhancement since augmented behavioral responses were observed during stimulation (acquisition). However, optogenetic stimulation of dopaminergic terminals from the VTA to the insular cortex does not modify the behavioral response (lack of neophobia) to the subthreshold stimuli while facilitating taste recognition memory performance during retrieval. It is essential to mention that optogenetic activation of VTA neurons or terminals does not alter the valence of the stimulus (Gil-Lievana et al., 2022), suggesting an increased effect over memory consolidation despite the low salient stimulus. Consequently, dopaminergic stimulation within the insular cortex enhances the perceptual salience for low-intensity stimulus. Similar results are observed after the optogenetic stimulation of LC-dopaminergic inputs into the hippocampus, which evokes dopamine release and enhances OLRM consolidation (Kempadoo et al., 2016).

Additionally, perceptual salience enhancement requires activation of the D1-like receptor. The blockade of cortical D1 receptors impairs long-term recognition memory keeping the short-term recognition memory intact. Conversely, administration of a D1 receptors agonist into the perirhinal cortex (SKF38393) before a subthreshold stimulation in a NOR protocol does not increase exploration times during acquisition. However, it induces NOR consolidation enhancement causing a clear novel object discrimination during the test (Balderas et al., 2013a). Although the blockade of D1-like receptors within the dorsal hippocampus spares NOR consolidation (Balderas et al., 2013a; Rossato et al., 2013). Recent studies report that the administration of dopamine or a D1 receptor agonist into the dorsal hippocampus enhances NOR persistence

TABLE 2 Pharmacological effects of catecholaminergic drugs on NOR and OLRM performance.

	Receptor	Drug	Mechanism	Time of administration	Task	Effect on memory	Reference
Intracerebroventricular							
	D1/D5	SCH-3390	Antagonist	Before training	Object-in-context	↓	Lemon and Manahan-Vaughan (2006)
Anterolateral hypothalamus							
	Dopamine and noradrenaline uptake transporter	6-OHDA	Neuotoxin	7 days before training	NOR	↓	Stephen Fink and Smith (1980)
Basolateral amygdala							
	β	Propranolol	Antagonist	Post-training	Object-in-context	↓	Barsegyan et al. (2014)
	α and β	Norepinephrine	Agonist	Post-training	Object-in-context	↑	Barsegyan et al. (2014)
	D1/D5	SCH-23390	Antagonist	Post-training	NOR	↓	Rossato et al. (2013)
Dorsal hippocampus							
	Dopamine and noradrenaline uptake transporter	6-OHDA	Neuotoxin	7 days before training	NOR	=	Moreno-Castilla et al. (2017)
	Dopamine and noradrenaline uptake transporter	6-OHDA	Neuotoxin	7 days before training	OLRM	↓	Moreno-Castilla et al. (2017)
	β	Timolol	Agonist	Post-training	NOR	↑	Mello-Carpes et al. (2016)
	α and β	Norepinephrine	Agonist	Post-training	NOR	↑	Mello-Carpes et al. (2016)
	D1/D5	SCH3390	Antagonist	After training	NOR	↓	Yang et al. (2017)
	D1/D5	SCH-23390	Antagonist	Post-training	NOR	=	Rossato et al. (2013)
	D1/D5	SCH-23390	Antagonist	Before training	NOR	=	Balderas et al. (2013a)
	D1/D5	SKF38393	Agonist	Before training	NOR	=	Balderas et al. (2013a)
Medial Prefrontal cortex							
	D1/D5	SCH-23390	Antagonist	Post-training	NOR	↓	Rossato et al. (2013)
Nucleus accumbens (Core or shell region)							
	Dopamine and noradrenaline uptake transporter	6-OHDA	Neuotoxin	7 days before training	OLRM	↓	Nelson et al. (2010)
Nucleus accumbens (Core region)							
	Dopamine and noradrenaline uptake transporter	6-OHDA	Neuotoxin	7 days before training	NOR	=	Nelson et al. (2010)
Perirhinal cortex							
	D1/D5	SCH23390	Antagonist	Before training	NOR	↓	Balderas et al. (2013a)
	D1/D5	SKF38393	Agonist	Before training	NOR	↑	Balderas et al. (2013a)
Prefrontal cortex							
	D1/D5	SKF81297	Agonist	Before training	NOR	↓	Pezze et al. (2015)
	D1	SCH-23390	Antagonist	Before training	NOR	↓	Nagai et al. (2007) and Rossato et al. (2013)
	D2	Raclopride	Antagonist	Before training	NOR	=	Nagai et al. (2007), Rossato et al. (2013), and Pezze et al. (2015)
VTA							
	GABA A	Muscimol	Agonist	Post-training	NOR	↓	Rossato et al. (2013)

↑ indicates enhanced memory, ↓ indicates impaired memory and = indicates spared memory.

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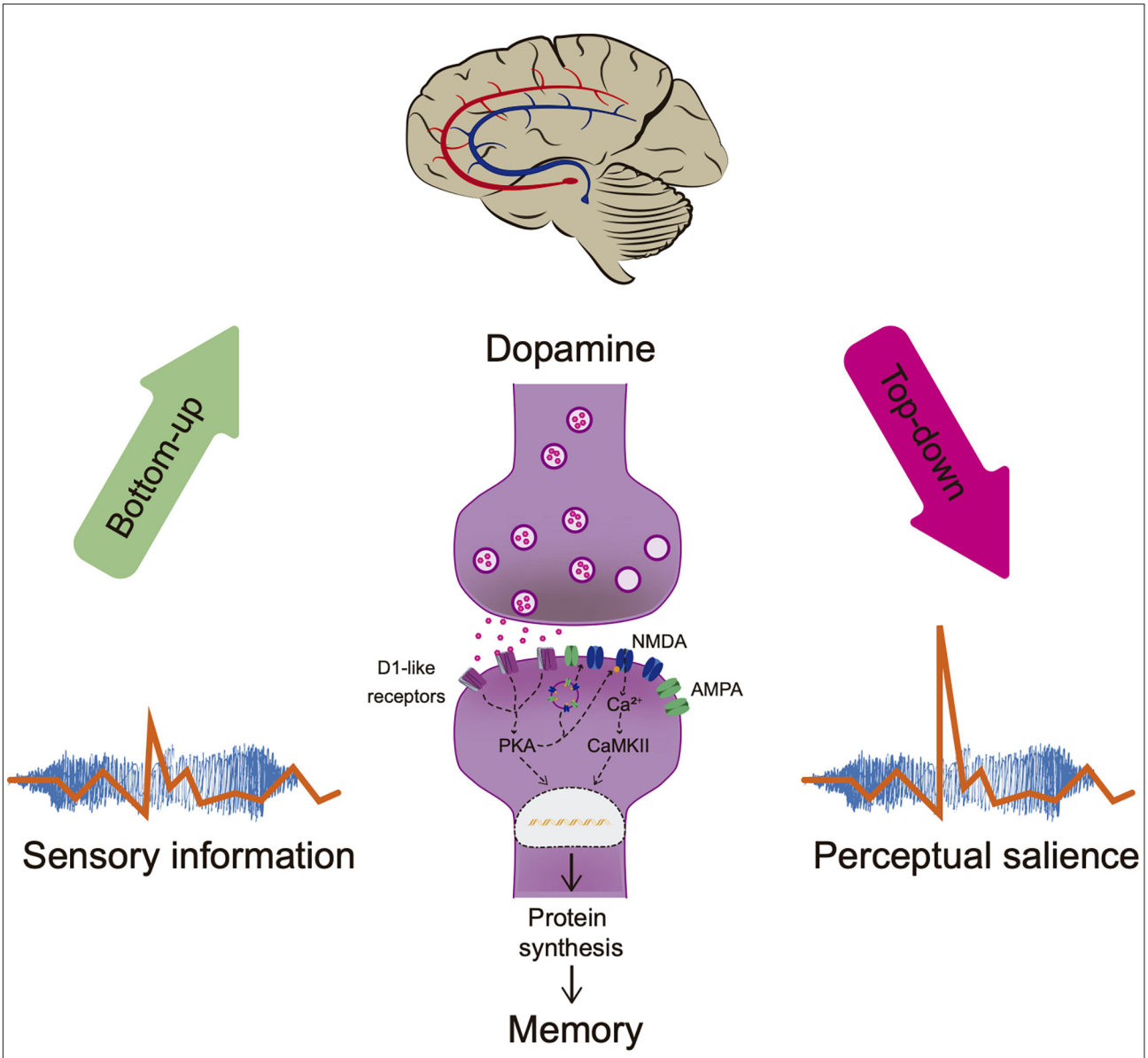

FIGURE 2
Perceptual salience. Sensorial stimulation attracts attentional resources bottom-up facilitating information processing. Brain hierarchizes salient information according to the physical stimuli properties and the organism's experience (meaning, consequences, or relationship with other stimuli in the environment). Dopamine regulates neuronal plasticity through activation of D1-like receptors enhancing memory consolidation, improving NMDA's conductance, inducing protein synthesis, and AMPA/NMDA receptors externalization. Memory modulates top-down processing without altering the initial sensory perception necessary for recognition by comparing the stimuli with the stored information and adjusting salience processing, a term referred to as perceptual salience. PKA, Protein Kinase A; Ca^{2+}, calcium; CaMKII, calcium calmodulin kinase.

(Vargas et al., 2020; Lima et al., 2022). These results suggest that the memory trace formation expressed in short-term memory is not dopamine-dependent, but its activity enables long-term and persistent storage (Balderas et al., 2013a; Moreno-Castilla et al., 2017; Vargas et al., 2020). In contrast, the blockade of D1-like receptor activity within the insular cortex impedes perceptual salience enhancement (Gil-Lievana et al., 2022). Hence, dopaminergic stimulation promotes the consolidation and persistence of stimuli that under normal conditions are not possible considering their subthreshold properties. In this regard, it has been suggested that dopaminergic responses are not related to signaling the stimuli's intensity but rather to the perceived intensity (de Lafuente and Romo, 2011). Although, these authors concluded that midbrain dopamine neurons code the subjective perception of the event (Romo and Rossi-Pool, 2020). We suggest that dopaminergic activity within the hippocampus, perirhinal and insular cortices facilitate the consolidation of information into long-term and persistent memories enabling the perceptual salience to guide behavior efficiently and effectively in future encounters. Considering that

dopamine modulates synaptic plasticity, dopaminergic signaling promotes the consolidation of inconspicuous stimulus into a relevant memory without altering the initial sensory perception.

Impact of catecholaminergic alterations in recognition memory

Dysregulation of dopaminergic signaling hinders declarative memories such as recognition memory (Guzmán-Ramos et al., 2012; Moreno-Castilla et al., 2017; Hernández-Ramírez et al., 2021). In some pathological brain conditions, such as schizophrenia, Alzheimer's, and Parkinson's diseases, the alteration in the catecholaminergic system leads to cognitive impairments. Schizophrenia is a mental disorder characterized by psychotic events that alter beliefs, perceptions, and emotions (Kapur, 2003). It has been demonstrated that over-activation of the dopaminergic pathways is associated with schizophrenia (Brisch et al., 2014; Winton-Brown et al., 2014). Consequently, dysregulated dopamine transmission leads to a stimulus-independent release of dopamine, generating an aberrant assignment of salience to external objects, exaggerating the perception (Kapur, 2003). Thus, aberrant perception salience involves attentional resources during irrelevant stimuli coding and drives cognitive processes inappropriately (Roiser et al., 2013). This distorted perception results from excess dopamine signaling within several brain areas, such as the ventral striatum, the prefrontal cortex. and the hippocampus (please see Kapur, 2003; Roiser et al., 2013). Noticeably, dopaminergic alterations in functional connectivity have been reported in the salience network, including dysfunctional connectivity in the anterior insular and anterior cingulate cortices, which correlates with excessive salience attributable to internal experiences (Rössler et al., 2020). In animal studies, systemic administration of methamphetamine hinders NOR due to dopaminergic system over-activation (Belcher et al., 2008; Herring et al., 2008; Camarasa et al., 2010; Razavi et al., 2020; Khodamoradi et al., 2022). Additionally, the dopamine transporter knockout mouse mimics specific symptoms observed in schizophrenia due to the increased dopaminergic activity; this mouse model also exhibits memory impairments, including NOR alterations (Wong et al., 2012). Most antipsychotic pharmacological treatments involve dopaminergic regulation. These drugs alleviate schizophrenia symptoms and ameliorate NOR deficits, the systemic administration of a D4 receptor agonist (Miyauchi et al., 2017), a D3 (Sun et al., 2016; Gou et al., 2017), and a D2 antagonist (McIntosh et al., 2013) improves the recognition for novel objects in animal models of schizophrenia mental disorder.

Alzheimer's disease is a progressive neurodegenerative disorder distinguished by the accumulation of amyloid β oligomers, plaques, and neurofibrillary tangles. Recent studies have suggested that the catecholaminergic system is affected during the first stages of the pathology. The neurodegeneration initiates within the LC (Braak and Del Tredici, 2015) and the VTA (Serra et al., 2018), propagating to the medial temporal lobe and cortical regions (Flores et al., 2022; Guzmán-Ramos et al., 2022), causing cognitive impairments. Our group reported that the accumulation of β-amyloid in a transgenic mouse model of Alzheimer's disease induces catecholaminergic neuronal loss (Moreno-Castilla et al., 2016). Importantly, in the same mouse model, animals exhibit NOR and OLRM impairments; these effects are attributable to a failure in dopamine release (Guzmán-Ramos et al., 2012; Moreno-Castilla et al., 2017). Moreover, stimulation of the dopaminergic system through the systemic administration of dopamine precursor levodopa (Ambrée et al., 2009) or a dopamine reuptake blocker (Guzmán-Ramos et al., 2012) attenuates NOR impairment observed in Alzheimer's disease mice models. Although memory deficits are strongly related to Alzheimer's disease, some patients exhibit sensorial alterations, including visual, olfactory, somatosensory, and auditory impairments (Mapstone et al., 2006; Daulatzai, 2016), probably due to early catecholaminergic alterations (Rey et al., 2012).

Parkinson's disease is another progressive neurodegenerative disorder related to the dysfunction of the catecholaminergic system. This pathology is characterized by several motor symptoms caused by aggressive dopaminergic cell loss in the substantia nigra (Lotharius and Brundin, 2002). Furthermore, Parkinson's disease patients also exhibit cognitive detriments (Aarsland, 2016). Administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a selective dopaminergic cell neurotoxin, into the substantia nigra is widely used as an animal model for Parkinson's disease; rats treated with this neurotoxin display degeneration of nigrostriatal dopaminergic neurons and NOR impairment (Sy et al., 2010; Chang and Wang, 2021). The administration of 6-OHDA into the striatum generates NOR (Chao et al., 2013; Masini et al., 2018) and OLRM deficits (Xie and Prasad, 2020; Barón-Quiroz et al., 2021). Moreover, the chronic treatment of reserpine, a monoamine-depleting agent, is also employed as a pharmacological model of Parkinson's disease in rodents; this animal model likewise shows long-term NOR memory impairment (Ikram and Haleem, 2019). A transgenic mice model of Parkinson's disease (MitoPark) resembles progressive neurodegeneration and death of dopaminergic neurons, loss of motor function, and deficits in NOR tasks (Li et al., 2013). This evidence shows that NOR and OLRM memory decline accompanies dopaminergic dysfunction in Parkinson's disease models. Although these Parkinson's disease models are suitable for emulating motor alterations, it is essential to mention that cognitive deficits in NOR and OLRM tasks appear before motor symptoms. Recognition memory impairments observed in Parkinson's disease could also be explained due to alterations in perception, visual hallucinations being the most frequently observed in Parkinson's patients (for a review, Russo et al., 2019); probably caused by alterations in the dopaminergic system.

Therefore, deficits in NOR and OLRM task performance exhibited in many animal models of brain disorders result from dopaminergic dysregulation. Catecholaminergic alterations reported as hypoactivation or hyperactivation disrupt the finely tuned dopaminergic system, probably negatively impacting the salience circuits involved in recognition memory. Thus, Alzheimer's, Parkinson's, schizophrenia, and other brain disorders related to altered catecholaminergic signaling might alter the integration of information required for perceptual salience and the subsequent cognitive processes such as memory, attention, and motivation.

Conclusions

Relevant information is more efficiently processed in comparison to non-relevant stimuli. Selection of relevant information from the environment is accomplished since intense physical properties are intrinsically salient. However, salience could also be modulated by "internal" factors according to the observer's experience. Hence, perceptual processing occurs according to the intense physical properties of the stimulus or the previously learned information. Former perceptual processing requires a close interaction between perception and memory, considering that integrated memories modulate perception and salience processing. Here, we propose the term perceptual salience to explain how memory mediates the integration of inconspicuous stimuli into a relevant memory trace, facilitating salience detection in future encounters without apparently altering the recognition of the physical attributes or valence of the stimuli.

Memory is a fundamental cognitive function that integrates information allowing recognition of familiar (non-salient) events from novel (salient) ones. In general, recognition memory involves acquiring, consolidating, retrieving, and updating two differential processes: recollection and familiarity. Recollection recovers the characteristics of the stimulus within a context, whereas familiarity integrates whether a stimulus is new or has already been experienced. Several brain regions integrate new information learning; the hippocampus, prefrontal cortex, parahippocampal (perirhinal, entorhinal, and postrhinal), and insular cortices are widely involved in recognition memory. Moreover, the catecholaminergic system modulates cognitive functions, including recognition memory. The main catecholaminergic inputs arise from the LC and the VTA, which innervate several brain structures related to recognition memory. Novel object and object location recognition memory are the most common behavioral paradigms employed to determine recognition memory's acquisition, consolidation, retrieval, and updating due to the natural tendency of rodents to explore novel stimuli. Exposure to novel stimuli or spatial configuration induces a dopamine release, modulating dopaminergic receptors that strengthen learning signals, and facilitating the transition of novelty to familiarity. Mainly, dopaminergic activity within the perirhinal and insular cortices and the hippocampus mediates the consolidation process of perceptual salience. Dopamine regulates plasticity-related events that enhance memory consolidation and persistence, regardless of the initial sensory perception, improving perceptual salience during recognition memory. Importantly, brain disorders caused by neurodegenerative diseases such as Alzheimer's or Parkinson's, metabolic disorders, or schizophrenia alter recognition memory due to dopaminergic dysfunction, probably related to the distortion in perceptual salience and the subsequent cognition processes.

Author contributions

The authors confirm contribution to the manuscript as follows: DO-G, KG-R, and FB-R conceptualized, reviewed, and edited the manuscript; preparation of draft manuscript by DO-G. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Object recognition tasks in rats: Does sex matter?

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Novelty recognition tasks based on object exploration are frequently used for the evaluation of cognitive abilities and investigation of neurobiological and molecular aspects of memory in rodents. This is an interesting approach because variations of the object recognition tasks focus on different aspects of the memory events such as novelty, location, context, and combinations of these elements. Nevertheless, as in most animal neuroscience research, female subjects are underrepresented in object recognition studies. When studies include females, the particularities of this sex are not always considered. For example, appropriate controls for manipulations conducted exclusively in females (such as estrous cycle verification) are not included. In addition, interpretation of data is often based on standardizations conducted with male subjects. Despite that, females are frequently reported as deficient and unable to adequately perform some memory tests. Thus, our study aims to review studies that describe similarities and differences between male and female performances in the different variations of object recognition tasks. In summary, although females are commonly described with deficits and the articles emphasize sex differences, most published data reveal similar performances when sexes are compared.

KEYWORDS

cognition, behavioral task, spatial memory, ovariectomy, vaginal lavage

Introduction

Historically, female subjects are neglected in biomedical science. Particularly, in neuroscience, over 5 males are used for each female, and the reason to avoid females is the alleged variation due to their reproductive cycles (Zucker and Beery, 2010). However, sexual features are relevant biological variables (National Institute of Health [NIH], 2015), and the inclusion of equal numbers of both the sexes in the studies is recommended. Female and male animals can exhibit completely different responses in the same behavioral task (Ribeiro et al., 2010). Therefore, we should not only include females, but be aware of the peculiarities of this sex. Specifically, there is a common sense that females do not perform as well as males in memory tasks (particularly in spatial memory) (Vorhees and Williams, 2014). In addition, most of the studies use procedures

to control or suppress the natural female hormone cycle, such as vaginal lavage procedure (VLP) or ovariectomy, regardless of the consequences of these manipulations, which are insufficiently studied. Even considering evidence that female's performance is worse, some of the reasons that could explain this fact beyond a cognitive difference *per se* are: (1) most, if not all, tasks are standardized for males; (2) the manipulations performed only in females could result in misinterpretation of data, if not controlled; and (3) publication bias, as both the authors and journals show a preference for publication of positive over negative results (sex differences over sex similarities). Thus, the equalization of the number of subjects between sexes is not enough. More attention should be paid to methods of including females, adequate controls, and interpretation of results without considering male's performance the "normal" one. Finally, it is important to consider comprehensive surveys of the literature when discussing sex comparisons or female behavior.

Four versions of object recognition's task are used in the studies selected for the present review: (1) Novel object recognition (NOR): rats are presented to 2 identical objects in the training session, and in the test session one object is changed for a new object; it is expected that the rat explores more the novelty (Abbott et al., 2016); (2) Place recognition: rats are presented to 2 identical objects in the training session, and in the test session one object is in a different position, which adds a spatial aspect to the task; it is expected that the rat explores more the moved object (Abbott et al., 2016); (3) Object-in-place recognition (OIPR): there are 4 different objects in the training session, and in the test session 2 of those objects exchange places; this version combines the spatial aspect with the object identification; it is expected that the rat explores more the reallocated objects (Abbott et al., 2016); and (4) Object-in-context recognition (OICR): rats are presented to 2 identical objects in a context A (for example, dark room and dark apparatus), then presented to 2 new identical objects in context B (for example, bright room, and bright apparatus); afterward, rats are placed in context A or B with 1 object of each context; it is expected that the rat explores more the object presented in a context different from the one it was first seen (Lee et al., 2014). There are other versions of object recognition tasks that have not been explored in female animals yet. For example, some protocols consider the order of objects presented as a temporal aspect of recognition memory (Barbosa et al., 2012).

It is known that sex and sex steroids impact recognition tasks, and that females' performance can differ from males in NOR tasks (McCarthy et al., 2018). Recognition tasks can be used in the study of diseases such as brain injuries, attention-deficit hyperactivity disorder, or Alzheimer's disease (which differs between sexes in several aspects—de Macêdo Medeiros and Silva, 2019). Moreover, these tasks are also relevant for studying functional neuroanatomy, aging, and the role of neurotransmitters, which reinforce the need for studying both the sexes (Ennaceur and Silva, 2018).

Our study aimed to review published articles that used object recognition tasks to verify sex similarities and differences. Besides the reduced number of studies that include females, we discuss possible constraints of the studies that can be crucial to the interpretation of females' behavior, such as manipulations that are exclusive to this sex. Therefore, we expect to incentivize the inclusion of both the sexes in object recognition studies, with adequate approaches to study female rats' behavior and compare performances between sexes.

Methods

The studies were selected using the PubMed database (accessed on 12 October 2021).[1] The search terms were "sex differences and object recognition and rat" and the filter for "other animals" was used. Articles that did not use rats, did not test males and females in the same task, considered the data of males and females together for analysis, were not clear about the sex of animals used, did not include an object recognition task, or did not include the control groups with no previous manipulation not related to the estrous cycle were excluded from the survey. Every article comparing male and female rats in a version of object recognition task with groups that had no previous manipulation was included.

Results

A preliminary search returned 6,662 articles when the term "object recognition" was combined with the PubMed filter "other animals." When we added the filter "females," 1,567 articles were listed, suggesting 23.52% of the articles in the first search included females. This percentage of studies, including females, may not look much, but in the field of neuroscience, the proportion is usually five males for each female (Zucker and Beery, 2010), revealing that articles on object recognition tasks are not particularly sex-biased.

The main search was conducted according to the detailed criteria described above, and 56 articles were selected (see **Figure 1** and **Table 1**). Most of the selected articles included groups submitted to manipulations not related to sex; those groups were not considered in our analysis.

Novel object recognition

Most articles revealed that female and male had similar performances considering discrimination ratio (Ennaceur et al., 2005; Salas-Ramirez et al., 2010; Muhammad et al., 2011;

1 https://pubmed.ncbi.nlm.nih.gov/

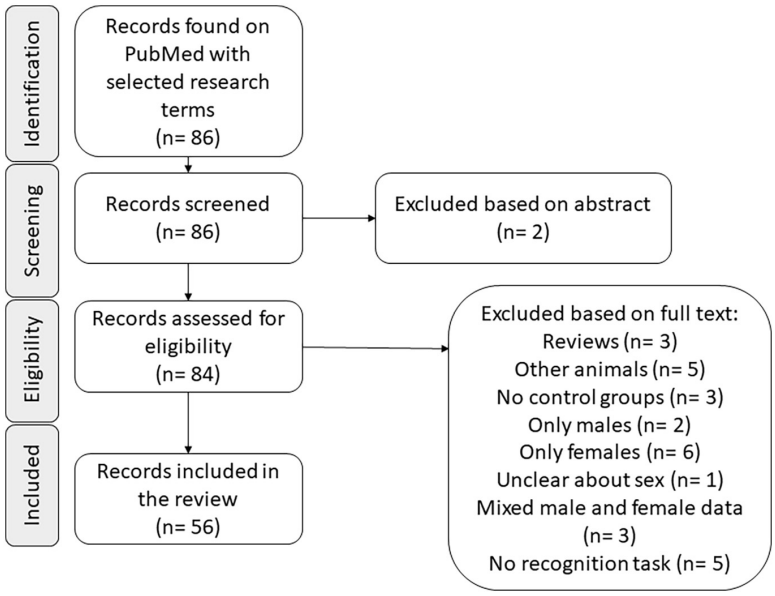

FIGURE 1
Graphical flow diagram of the article selection process (n: number of articles in each phase).

Muhammad and Kolb, 2011; Fielding et al., 2012; Howland et al., 2012; Klug and van den Buuse, 2012; Mansouri et al., 2012; van Goethem et al., 2012; Zamberletti et al., 2012; Kolyaduke and Hughes, 2013; Marco et al., 2013; Hill et al., 2014; Abbott et al., 2016; Alteba et al., 2016; Anselmi et al., 2016; Barbie-Shoshani et al., 2016; Turgeon et al., 2016; Arfa-Fatollahkhani et al., 2017; Bengoetxea et al., 2017; Jordan and Andersen, 2018; Lian et al., 2018; Winther et al., 2018; Bruijnzeel et al., 2019; Klambatsen et al., 2019; Sadegzadeh et al., 2020), percentage of time—relative time exploring the novel object considering total amount of object exploration (Pereira et al., 2008; Bowman et al., 2009, 2015; van Goethem et al., 2012; Nelson et al., 2018; Ellis et al., 2020; Macht et al., 2020; Gillera et al., 2021; Peay et al., 2021), or absolute time—duration of novel object exploration (Paris and Frye, 2011; Reichel et al., 2012; Weston et al., 2014; Gonzales et al., 2015; Braun et al., 2018; Santollo et al., 2019; Villanueva Espino et al., 2020).

Some studies demonstrated that females were better than males, based on one of these outcomes: only females preferred the novelty (Salomon et al., 2011; Sallaberry et al., 2018), females learned regardless of housing, while only single-housed males (Beck and Luine, 2002), females learned even if exploring less the objects during training (Mourlon et al., 2010; Robison et al., 2017; Wooden et al., 2021), females made more visits and spent more time exploring the novelty (Foley et al., 2014), or females retained the memory for a longer period (Ghi et al., 1999; Sutcliffe et al., 2007). On the other hand, some studies showed that males were better than females due to the following results: males retained the memory for a longer period (Baran et al., 2010), only males learned when aged 40 days, while both the sexes learned at other ages (Cyrenne and Brown, 2011a DevP), or males had higher discrimination indexes (Cyrenne and Brown, 2011b).

Place recognition

Most articles revealed that females and males had similar performances considering the discrimination ratio (Ennaceur et al., 2005; Baran et al., 2010; Salas-Ramirez et al., 2010; Abbott et al., 2016; Alteba et al., 2016) and the percentage of time exploring objects (Bowman et al., 2015; Peay et al., 2021). Some studies concluded that males were better than females because females did not differentiate the objects (Beck and Luine, 2002; Bowman et al., 2009), only estrus females learned (Sutcliffe et al., 2007), or males had higher discrimination indexes (Howland et al., 2012).

Object-in-place recognition

Most articles revealed that females and males had similar performances considering the discrimination ratio (Howland et al., 2012; Abbott et al., 2016) and absolute time (Reichel et al., 2012). One of the studies demonstrated that females did not discriminate the objects when submitted to VLP, but learns when submitted to ovariectomy, while intact and unstressed males learned the task (Cost et al., 2012). Another study demonstrated that male and female rats differentiated the

TABLE 1 Summarized information of the selected articles.

References	Title	Strain (age during the test)	Manipulations that could have impacted sex comparisons	Recognition task	Outcome of control animals
Abbott et al., 2016	Sex-specific effects of daily exposure to sucrose on spatial memory performance in male and female rats, and implications for estrous cycle stage	Sprague Dawley (3 months old)	VLP	Novel object	Both male and female differentiated the objects
				Place	Both male and female differentiated the objects
				Object-in-place	Both male and female differentiated the objects
Arfa-Fatollahkhani et al., 2017	The effect of luteinizing hormone reducing agent on anxiety and novel object recognition memory in gonadectomized rats	Wistar (4 months old)	OVX + HR and unclear about VLP	Novel object	Both male and female differentiated the objects
Alteba et al., 2016	Cannabinoids reverse the effects of Early stress on neurocognitive performance in adulthood	Unclear (20 days old)	Unclear	Novel object	Both male and female differentiated the objects
				Place	Both male and female differentiated the objects
Anselmi et al., 2016	Genetic evidence for chromosome 4 loci influencing learning and memory	Sprague Dawley, LEW and SHR (after 11 weeks old)	Unclear	Novel object	Both male and female differentiated the objects
Braun et al., 2018	Sex-specific effects of Cacna1c haploinsufficiency on object recognition, spatial memory, and reversal learning capabilities in rats	Sprague Dawley (94 day old)	Unclear	Novel object	Both male and female differentiated the objects
Barbie-Shoshani et al., 2016	Sex-specific effects of prenatal stress on memory and markers of neuronal activity in juvenile rat	Wistar (24–32 days old)	Unclear	Novel object	Both male and female differentiated the objects
Bengoetxea et al., 2017	Effects of perinatal diet and prenatal stress on the behavioral profile of aged male and female rats	Wistar (1 and 19 months old)	Unclear	Novel object	Both male and female differentiated the objects
Beck and Luine, 2002	Sex differences in behavioral and neurochemical profiles after chronic stress: Role of housing conditions	Sprague Dawley (50–60 days old)	Unclear	Novel object	Females learned regardless of housing
				Place	Only males learned
Bowman et al., 2006	Aged rats: Sex differences and responses to chronic stress	Sprague Dawley (20 months old)	Unclear	Novel object	Both male and female differentiated the objects
				Place	Nor male or female differentiated the objects, not included
Baran et al., 2010	Prefrontal cortex lesions and sex differences in fear extinction and perseveration	Sprague Dawley (age unspecified, weight 275–300 g)	VLP	Novel object	Males retained the memory for longer
				Place	Both male and female differentiated the objects
Bowman et al., 2009	Sex-dependent changes in anxiety, memory, and monoamines following 1 week of stress	Sprague Dawley (8 weeks old)	Unclear	Novel object	Both male and female differentiated the objects
				Place	Only males learned
Bruijnzeel et al., 2019	Effects in rats of adolescent exposure to cannabis smoke or THC on emotional behavior and cognitive function in adulthood	Long Evans (129 days old)	Unclear	Novel object	Both male and female differentiated the objects
Bowman et al., 2015	Bisphenol-A exposure during adolescence leads to enduring alterations in cognition and dendritic spine density in adult male and female rats	Sprague Dawley (5 weeks old)	Unclear	Novel object	Both male and female differentiated the objects
				Place	Both male and female differentiated the objects
Cost et al., 2012	Sex differences in object-in-place memory of adult rats	Long evans (55–60 days old)	VLP or OVX + HR	Object-in-place	Females submitted to VLP didn't learn
Cyrenne and Brown, 2011a	Ontogeny of sex differences in response to novel objects from adolescence to adulthood in lister-hooded rats	Lister hooded (28–80 days old)	Unclear	Novel object	Males learned in every age tested, females didn't learn by the age of 40 day
Cyrenne and Brown, 2011b	Effects of suppressing gonadal hormones on response to novel objects in adolescent rats	Lister hooded (40 days old)	Unclear	Novel object	Both male and female differentiated the objects, males had higher preference

(Continued)

TABLE 1 Continued

References	Title	Strain (age during the test)	Manipulations that could have impacted sex comparisons	Recognition task	Outcome of control animals
Ellis et al., 2020	Paternal morphine self-administration produces object recognition memory deficits in female, but not male offspring.	Sprague Dawley (age unspecified, weight 250–300 g)	Unclear	Novel object	Both male and female differentiated the objects
				Place	Females learned, males were not tested, not included
Ennaceur et al., 2005	Detailed analysis of the behavior of lister and Wistar rats in anxiety, object recognition and object location tasks	Long evans and Wistar (2 months old)	Unclear	Novel object	Both male and female differentiated the objects
				Place	Both male and female differentiated the objects
Fielding et al., 2012	Profiles of motor and cognitive impairment in the transgenic rat model of Huntington's disease	Sprague Dawley (22 months)	Unclear	Novel object	Both male and female differentiated the objects
Foley et al., 2014	Sexually dimorphic effects of prenatal exposure to propionic acid and lipopolysaccharide on social behavior in neonatal, adolescent, and adult rats: Implications for autism spectrum disorders	Long evans (43 days old)	Unclear	Novel object	Both male and female differentiated the objects, females made more visits to the objects, and spent more time with the objects
Gillera et al., 2021	Sex-specific effects of Perinatal FireMaster® 550 (FM 550) exposure on socioemotional behavior in prairie voles	Unclear (80 days old)	Unclear	Novel object	Both male and female differentiated the objects
Ghi et al., 1999	Sex differences in memory performance in the object recognition test. Possible role of histamine receptors	Wistar (40 days old)	Unclear	Novel object	Females retained the memory for longer
Gonzales et al., 2015	Repeated neonatal propofol administration induces sex-dependent long-term impairments on spatial and recognition memory in rats.	Kyoto Wistar (6 weeks old)	Unclear	Novel object	Both male and female differentiated the objects
Howland et al., 2012	Altered object-in-place recognition memory, prepulse inhibition, and locomotor activity in the offspring of rats exposed to a viral mimetic during pregnancy	Long evans (60–90 days old)	VLP	Novel object	Both male and female differentiated the objects
				Place	Both male and female differentiated the objects, males had higher preference
				Object-in-place	Both male and female differentiated the objects
Hill et al., 2014	Sex-specific disruptions in spatial memory and anhedonia in a "two hit" rat model correspond with alterations in hippocampal brain-derived neurotrophic factor expression and signaling	Wistar (6 weeks old)	Unclear	Novel object	Both male and female differentiated the objects
Jordan and Andersen, 2018	Working memory and salivary brain-derived neurotrophic factor as developmental predictors of cocaine seeking in male and female rats	Sprague Dawley (20 days)	None	Novel object	Both male and female differentiated the objects
Klambatsen et al., 2019	Sex differences in memory and intracellular signaling after methamphetamine binge treatment	Sprague Dawley (8 weeks old)	OVX and unclear about VLP	Novel object	Both male and female differentiated the objects
Kolyaduke and Hughes, 2013	Increased anxiety-related behavior in male and female adult rats following early and late adolescent exposure to 3,4-methylenedioxymethamphetamine (MDMA)	PVG/C hooded (90 days old)	Unclear	Novel object	Both male and female differentiated the objects
Klug and van den Buuse, 2012	Chronic cannabinoid treatment during young adulthood induces sex-specific behavioral deficits in maternally separated rats	Wistar (8 weeks old)	Unclear	Novel object	Both male and female differentiated the objects
Lian et al., 2018	Object, spatial and social recognition testing in a single test paradigm.	Sprague Dawley (age unspecified, weight 170–200 g)	Unclear	Novel object	Both male and female differentiated the objects
				Place	Both male and female differentiated the objects

(Continued)

TABLE 1 Continued

References	Title	Strain (age during the test)	Manipulations that could have impacted sex comparisons	Recognition task	Outcome of control animals
Macht et al., 2020	Adolescent alcohol exposure produces protracted cognitive-behavioral impairments in adult male and female rats	Long evans (9 months old)	Unclear	Novel object	Both male and female differentiated the objects
Mansouri et al., 2012	Gender-dependent behavioral impairment and brain metabolites in young adult rats after short term exposure to lead acetate	Wistar (55–60 days old)	Unclear	Novel object	Both male and female differentiated the objects
Marco et al., 2013	Maternal deprivation effects on brain plasticity and recognition memory in adolescent male and female rats	Wistar (after 22 days old)	Unclear	Novel object	Both male and female differentiated the objects
Muhammad and Kolb, 2011	Mild prenatal stress-modulated behavior and neuronal spine density without affecting amphetamine sensitization	Long evans (30–40 days old)	Unclear	Novel object	No effect of sex
Muhammad et al., 2011	Tactile stimulation during development attenuates amphetamine sensitization and structurally reorganizes prefrontal cortex and striatum in a sex-dependent manner	Long evans (30–40 days old)	Unclear	Novel object	Both male and female differentiated the objects
Mourlon et al., 2010	Maternal deprivation induces depressive-like behaviors only in female rats	Long evans (68–111 days old)	Unclear	Novel object	Both male and female differentiated the objects, female would learn exploring less the objects during the training phase
Nelson et al., 2018	Chronic moderate alcohol drinking alters insulin release without affecting cognitive and emotion-like behaviors in rats	Long evans (23 days old)	Unclear	Novel object	Both male and female differentiated the objects
Pereira et al., 2008	Early enriched housing results in partial recovery of memory deficits in female, but not in male, rats after neonatal hypoxia-ischemia	Wistar (30 days old)	Unclear	Novel object	Both male and female differentiated the objects
Paris and Frye, 2011	Juvenile offspring of rats exposed to restraint stress in late gestation have impaired cognitive performance and dysregulated progestogen formation	Long evans (28–30 days old)	Unclear	Novel object	Both male and female differentiated the objects
Peay et al., 2021	Chronic unpredictable intermittent restraint stress disrupts spatial memory in male, but not female rats	Sprague Dawley (age unspecified, weight 200–225 g)	Unclear	Novel object	Both male and female differentiated the objects
				Place	Both male and female differentiated the objects
Reichel et al., 2012	Sex differences in escalation of methamphetamine self-administration: Cognitive and motivational consequences in rats	Long evans (age unspecified, males' weight 250–300 g, females' 180–200 g)	Unclear	Novel object	Both male and female differentiated the objects
				Object-in-place	Both male and female differentiated the objects
Robison et al., 2017	Sex differences in the physiological and behavioral effects of chronic oral methylphenidate treatment in rats	Sprague Dawley (4 weeks old)	Unclear	Novel object	Both male and female differentiated the objects, female would learn exploring less the objects during the training phase
Saucier et al., 2008	Sex differences in object location memory and spatial navigation in long-evans rats.	Long evans hooded (50 days old	Unclear	Object-in-place	Both male and female differentiated the objects, female would learn exploring less the objects during the training phase
Salomon et al., 2011	Corticosterone mediates some but not other behavioral changes induced by prenatal stress in rats	Wistar (31 days old)	VLP	Novel object	Only females learned
Salas-Ramirez et al., 2010	Prenatal cocaine exposure increases anxiety, impairs cognitive function and increases dendritic spine density in adult rats: influence of sex	Sprague Dawley (64–68 days old)	VLP	Novel object	Both male and female differentiated the objects
				Place	Both male and female differentiated the objects
Santollo et al., 2019	Gonadal hormones in female rats protect against dehydration-induced memory impairments in the novel object recognition paradigm	Sprague Dawley (age unspecified, weight 75–100 g)	VLP or OVX	Novel object	Both male and female differentiated the objects

(Continued)

TABLE 1 Continued

References	Title	Strain (age during the test)	Manipulations that could have impacted sex comparisons	Recognition task	Outcome of control animals
Sallaberry et al., 2018	Sex differences in the effects of pre- and post-natal caffeine exposure on behavior and synaptic proteins in pubescent rats	Wistar (35 and 70 days old)	Unclear	Novel object	Only females learned
Sadegzadeh et al., 2020	Effects of adolescent administration of fluoxetine on novel object recognition memory, anxiety-like behaviors, and hippocampal brain-derived neurotrophic factor level	Wistar (2–3 months old)	Unclear	Novel object	Both male and female differentiated the objects
Sutcliffe et al., 2007	Influence of gender on working and spatial memory in the novel object recognition task in the rat	Hooded lister (age unspecified, weight 234–373 g)	VLP	Novel object	Females retained the memory for longer
				Place	Estrous cycle's phases interfered in female behavior
Turgeon et al., 2016	Chronic caffeine produces sexually dimorphic effects on amphetamine-induced behavior, anxiety and depressive-like behavior in adolescent rats	Sprague Dawley (44 days old)	Unclear	Novel object	Both male and female differentiated the objects
van Goethem et al., 2012	Object recognition testing: Rodent species, strains, housing conditions, and estrous cycle	Wistar (4 months old)	VLP	Novel object	Both male and female differentiated the objects
Villanueva Espino et al., 2020	Cognitive training increases dendritic arborization in the dorsal hippocampal CA1 and CA3 neurons of female and male Long–Evans rats	Long evans (56 days old)	Unclear	Novel object	Both male and female differentiated the objects
Weston et al., 2014	Sex-dependent and Non-monotonic enhancement and unmasking of methylmercury neurotoxicity by prenatal stress	Long evans (3 months old)	Unclear	Novel object	Both male and female differentiated the objects
Wooden et al., 2021	A sensitive homecage-based novel object recognition task for rodents	Long Evans (70 days old)	Unclear	Novel object	Both male and female differentiated the objects, female would learn exploring less the objects during the training phase
Winther et al., 2018	Maternal high-fat diet programs offspring emotional behavior in adulthood	Sprague Dawley (7 weeks old)	Unclear	Novel object	Both male and female differentiated the objects
Zamberletti et al., 2012	Gender-dependent behavioral and biochemical effects of adolescent delta-9-tetrahydrocannabinol in adult maternally deprived rats	Sprague Dawley (65 days old)	Unclear	Novel object	Both male and female differentiated the objects

VLP, vaginal lavage procedure; OVX, ovariectomy; HR, hormonal reposition.

objects, despite females exploring less the objects during training (Saucier et al., 2008).

Object-in-context recognition

Although we did not find studies comparing male and female animals in OIPR tasks, an article using only females revealed that control rats learn this task considering discrimination index and time exploring the objects (Sasaki Russell et al., 2019).

Discussion

Most published articles revealed similar performances, but some articles suggested that females performed NOR better than males, and males performed PR better than females. Although this evidence is not robust considering all the studies together, these findings corroborate human studies in which females are better in object or color recognition and males are better in location recognition (McGivern et al., 2019). Regarding OIPR, literature does not show any sex as having better performance (see **Table 2**).

Some methodological aspects can hinder the collective interpretation of the selected articles: (1) the use of the discrimination index (also referred to as ratio). This parameter is commonly understood as (time exploring new object–time exploring old object)/(total exploration time), but many studies claim to use a discrimination index, but actually reported the percentage of time exploring the object, which can be confusing and makes it harder to compare the data; (2) many articles were not clear about the age of animals, which can lead to variability in the behavior; (3) the interpretation of the researchers is likely

TABLE 2 Number of articles revealing no differences, benefiting males, and benefiting females.

	Frequency	Percentage
Novelty object recognition		
No differences	41	78%
Benefits males	3	6%
Benefits females	9	16%
Total	54	100%
Place recognition		
No differences	9	69%
Benefits males	4	31%
Benefits females	0	0%
Total	13	100%
Object-in-place recognition		
No differences	3	60%
Benefits males	1	20%
Benefits females	1	20%
Total	5	100%

For all the three tasks, most published articles did not show any differences between female and male behaviors.

to consider non-significant data or tendencies that benefit males such as the time spent near the objects (Ceccarelli et al., 2001) or emphasizing females exhibited a lower discrimination index when they learn the task (Cyrenne and Brown, 2011b) or when neither male nor female spent more time exploring the new object (Bowman et al., 2006); and (4) even when there is a significant difference, most articles do not include the effect size in order to highlight the relevance of the behavioral difference.

Importantly, the absence of control groups in many published articles is a relevant issue, especially considering females' performances. Similar to what is done for male animals, the female control group must be free of specific stressors and manipulations, i.e., studies should include a group of female rats that are not submitted to VLP or ovariectomy/hormonal reposition. Indeed, many animals had been previously submitted to these manipulations, without including female rats that did not go through those procedures. In addition, most articles did not make it clear if they used these manipulations or how they evaluated the consequences of that use (Klambatsen et al., 2019; Santollo et al., 2019). Some of them used females submitted to VLP as controls and compared them to intact males and gonadectomized females (Cost et al., 2012). In this respect, it has been shown that estrous cycle monitoring is stressful (Becegato et al., 2021) and alters female behavior (Walker et al., 2002; Becegato et al., 2021). Only one of the selected articles highlighted that they avoided VLP because of the possibility of altering behavior (Jordan and Andersen, 2018). Another study using mice assessed the estrous cycle using the visual method daily and performed a single VLP to confirm the phase (Mitra et al., 2017), as proposed by Walker et al. (2002). Thus, few researchers that studied object recognition have shown adequate approaches to deal with particularities of studying behavior in females. Many studies compare stressed females (caused by VLP) to unstressed males, whereas it is well known that stress has a major impact on spontaneous behavior (Klenerová et al., 2007; Rabelo-da-Ponte et al., 2019) and memory (for a review, see Cazakoff et al., 2010). Thus, this is a major weakness of these studies. Hence, we suggest the addition of a control group of naïve females, which are not submitted to any manipulations regarding their hormonal fluctuations; in the same way, intact males are usually included as controls.

Two of the articles selected have included both the females that were monitored with VLP and ovariectomized females. In Cost et al.'s (2012) article, those female groups were compared to intact males in the OIPR task. The results showed that females that were submitted to VLP and tested in the diestrus phase had worse performance (decreased delay of retention) compared to males, while vehicle-treated ovariectomized females had similar performance compared to males. In Santollo et al.'s (2019) study, cycling females were compared to intact males and gonadectomized females in the NOR task. In one of the experiments, VLP females tested in the diestrus or estrus cycle presented performance comparable to males. In another experiment, the behavior of intact and ovariectomized females was similar, but it is not clear if intact females were submitted to estrous cycle monitoring. In another study, Klambatsen et al. (2019) compared ovariectomized females and intact females in the NOR task; both the groups differentiated the objects and spent a larger percentage of time with the novel object, but it is not clear if intact females were submitted to estrous cycle monitoring (Klambatsen et al., 2019).

A few articles evaluated the possible influence of the estrous cycle's phases on the performance of female rats. It has been shown that metestrus and diestrus females learned PR and OIPR tasks (Abbott et al., 2016). However, it has also been shown that only estrus rats preferred the moving object in the PR task (Sutcliffe et al., 2007) and that diestrus females only retained the memory of OIPR for 5 min (Cost et al., 2012). Regarding the NOR task, rats in all the phases showed adequate performance (Sutcliffe et al., 2007; van Goethem et al., 2012), but metestrus and diestrus animals had smaller discrimination indexes compared to proestrus and estrus animals (van Goethem et al., 2012). Thus, the differences and similarities in females' behavior across the estrous cycle are still unclear. Importantly, as mentioned, monitoring the estrous cycle involves a stressful procedure that could interact with the hormonal status to influence behavior. Overall, most of the articles were not clear about the evaluation of the estrous cycle's phases, and the ones that presented those data were far from unanimous. Importantly, the manipulations used to evaluate the estrous cycle phase can alter rat's behavior and even mask existing differences between the phases (Walker et al., 2002). On

the other hand, ovariectomy does not seem to impair NOR and OIPR tasks (Cost et al., 2012; Arfa-Fatollahkhani et al., 2017; Klambatsen et al., 2019).

It is relevant to highlight that most published articles did not describe the details of ovariectomy or VLP, which makes reproducibility difficult. For example, some articles did not inform the method chosen for estrous cycle monitoring (Salas-Ramirez et al., 2010; Salomon et al., 2011; Santollo et al., 2019). Frequently, it was not clear how many times VLP was performed (Baran et al., 2010; van Goethem et al., 2012), and post-surgical care was not always well described (Arfa-Fatollahkhani et al., 2017). In addition, sometimes ovariectomy surgery is barely cited (Klambatsen et al., 2019). Few articles had a simple but reasonable explanation for their methods choice (Cost et al., 2012; Abbott et al., 2016). These methodological description constraints involving VLP and ovariectomy can lead to difficulties in the interpretation of the studies, and the differences or similarities described in **Table 2** might be unrealistic.

It is relevant to point out the relevance of the terms sex and gender when performing literature surveys. Gender refers to the social roles, socialization, and expressions, and, hence, applicable only to human studies. In animal studies, sex should be used, as it refers to biological aspects such as chromosomes, genes, hormones, gonads, and genitals. Nevertheless, as this conceptualization is somewhat recent, some published articles use "gender" when referring to animals (Sutcliffe et al., 2007).

Finally, there are recent articles that still are not clear about the sex of the animals used or mix male and female data without a reasonable justification. An adequate form of mixing data from both the sexes is the work by Arbogast et al. (2019). They planned a cohort with male and female animals in a 50:50 sex ratio; then, they first evaluated the performances separately. Since no significant sex differences were found, they mixed the data of both the sexes. Authors should provide accurate descriptions of all aspects of the methods used in the studies.

In conclusion, the present literature review raises several aspects of object recognition studies with female subjects that can lead to flawed interpretations, such as the consideration of non-significant data that benefit males, the absence of appropriate control groups, and the use of manipulations that interfere with female physiology and behavior without considering these effects. However, even with those confounding factors, most data show that females learn all the types of recognition tasks and most data reveal no sex differences in the performance of these tasks. This outcome not only highlights the importance of including females in behavioral studies, but also indicates that comprehensive reviews can be important tools to discuss and interpret sex differences in neuroscience.

Author contributions

MB collected the data, performed the analysis, and wrote the study. RS coordinated the study and revised the manuscript. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cholinergic regulation of object recognition memory

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Object recognition memory refers to a basic memory mechanism to identify and recall various features of objects. This memory has been investigated by numerous studies in human, primates and rodents to elucidate the neuropsychological underpinnings in mammalian memory, as well as provide the diagnosis of dementia in some neurological diseases, such as Alzheimer's disease and Parkinson's disease. Since Alzheimer's disease at the early stage is reported to be accompanied with cholinergic cell loss and impairment in recognition memory, the central cholinergic system has been studied to investigate the neural mechanism underlying recognition memory. Previous studies have suggested an important role of cholinergic neurons in the acquisition of some variants of object recognition memory in rodents. Cholinergic neurons in the medial septum and ventral diagonal band of Broca that project mainly to the hippocampus and parahippocampal area are related to recognition memory for object location. Cholinergic projections from the nucleus basalis magnocellularis innervating the entire cortex are associated with recognition memory for object identification. Especially, the brain regions that receive cholinergic projections, such as the perirhinal cortex and prefrontal cortex, are involved in recognition memory for object-in-place memory and object recency. In addition, experimental studies using rodent models for Alzheimer's disease have reported that neurodegeneration within the central cholinergic system causes a deficit in object recognition memory. Elucidating how various types of object recognition memory are regulated by distinct cholinergic cell groups is necessary to clarify the neuronal mechanism for recognition memory and the development of therapeutic treatments for dementia.

KEYWORDS

basal forebrain, cholinergic system, hippocampus, muscarinic receptor, nicotinic receptor, perirhinal cortex

Introduction

Recognition memory is a simple type of declarative memory, defined as the ability to feel familiarity and to discriminate familiar items from unfamiliar ones (Mandler, 1980; Mackintosh, 1987; Squire, 1998). To evaluate recognition memory, spontaneous object recognition memory tasks are widely used in rodents (Ennaceur and Delacour, 1988;

Dere et al., 2006; Aggleton and Nelson, 2020). *In such tasks*, animals are placed in an apparatus with objects, and they explore spontaneously. When object recognition memory is normally preserved, the time spent exploring novel objects is longer than that spent exploring familiar objects. This novelty preference is derived from the innate behavior of rodents to react to what was changed.

Previous studies have included experiments with numerous variants of the object recognition memory task to elucidate its neuronal mechanisms of recognition memory (Brown and Aggleton, 2001; Squire et al., 2007). Lesion studies showed that recognition memory for object location depends on the hippocampus and entorhinal cortex but not on the perirhinal cortex (Save et al., 1992; Parron et al., 2006). The suppression of the perirhinal cortex caused impairment in recognition memory for object identification, whereas the hippocampal lesion did not impair that memory (Save et al., 1992; Abe and Iwasaki, 2001; Brown et al., 2012). In addition, the medial temporal lobe is one of the brain regions that receive projections from cholinergic neurons in the basal forebrain (Bigl et al., 1982; Mesulam et al., 1983; Rye et al., 1984). Functional cooperation among the medial temporal lobe structures pivotally functions in several aspects of object recognition memory (Brown and Aggleton, 2001; Squire et al., 2007; Aggleton et al., 2012).

Clinical studies also suggest that the dysfunction of the basal forebrain cholinergic system causes impairment in recognition memory. Alzheimer's disease is a severe memory disorder that is associated with a loss of cholinergic neurons in the forebrain, followed by neurodegeneration of a wide range of brain regions (Davies and Maloney, 1976; Pákáski and Kálmán, 2008; Schmitz and Zaborszky, 2021). The earliest sign of this disease is impairment in recognition of previously encountered stimuli (Ally, 2012). Cholinergic involvement in object recognition memory has been suggested by this clinical indication from Alzheimer's disease. However, it remains unclear how the distinct cell groups in cholinergic systems are involved in the memory and interact with each other.

In the present review, we describe cholinergic regulation of object recognition memory, in which different cholinergic cell groups in the basal forebrain contribute to different aspects of memory. We also explain several behavioral factors that affect the performance in the memory task. Finally, we discuss the therapeutic possibility of cholinergic agents for correction of the impairment of object recognition memory seen in dementia.

Central cholinergic system

In the central nervous system, cholinergic neurons are composed of several distinct cell groups (Mesulam et al., 1983; Woolf et al., 1984; Woolf and Butcher, 1985; see **Figure 1**). Basal forebrain cholinergic neurons provide their projections to the entire neocortex and limbic cortex (Schmitz and Zaborszky, 2021). Cholinergic interneurons make local innervations within the striatum and neocortex (Mesulam et al., 1983; Zhou et al., 2002; von Engelhardt et al., 2007). In the cholinergic system, acetylcholine acts on nicotinic and muscarinic acetylcholine receptors, which are ionotropic and G protein-coupled metabotropic receptors, respectively (Levey et al., 1991; Alkondon and Albuquerque, 2004; Dani and Bertrand, 2007). These types of receptors are differentially distributed in the hippocampus, neocortex, and striatum in presynaptic and postsynaptic manners (Dannenberg et al., 2017; Obermayer et al., 2017).

Cholinergic neurons in the basal forebrain are divided into several groups; the medial septum (MS), ventral/horizontal diagonal band of Broca (vDB/hDB), and nucleus basalis magnocellularis or nucleus basalis of Meynert (nBM). The MS and vDB include cholinergic neurons projecting mainly to the hippocampus (the CA1-CA3, hilus, and dentate gyrus) and subiculum *via* the fornix. They also provide cholinergic innervations to the entorhinal, perirhinal, postrhinal, retrosplenial, infralimbic and prelimbic cortices (Gaykema et al., 1990; Gulyás et al., 1999; Kondo and Zaborszky, 2016). Cholinergic signaling in these projection areas has been assumed to occur both non-synaptically and synaptically (Vizi and Kiss, 1998; Zoli et al., 1999; Takács et al., 2018). Cholinergic neurons located in the hDB, innervate the main olfactory bulb, insular cortex and piriform cortex (Woolf et al., 1984; Záborszky et al., 1986). The caudal part of the basal forebrain cholinergic system consists of large cholinergic neurons in the nBM. This group includes cholinergic cells that are distributed throughout the ventral pallidum, magnocellular preoptic nucleus, nucleus basalis and substantia innominate. This cell group innervates the entire neocortex (isocortex) and amygdala (Mesulam et al., 1983; Eckenstein et al., 1988). They also innervate allocortical areas including the retrosplenial, entorhinal, and perirhinal cortices (Bigl et al., 1982; Woolf and Butcher, 1982, 1985; Rye et al., 1984; Woolf et al., 1984; Carlsen et al., 1985; Woolf, 1991).

Various types of cholinergic system controlling object recognition memory

Cholinergic projections from the medial septum and ventral diagonal band of Broca

Previous studies have revealed that cholinergic neurons in the MS/vDB are important in certain types of object recognition memory. A cholinergic lesion in the MS with 192 IgG-saporin decreases choline acetyltransferase activity in the hippocampus and frontal cortex, and impairs object location memory, but not object recognition memory (Cai et al., 2012).

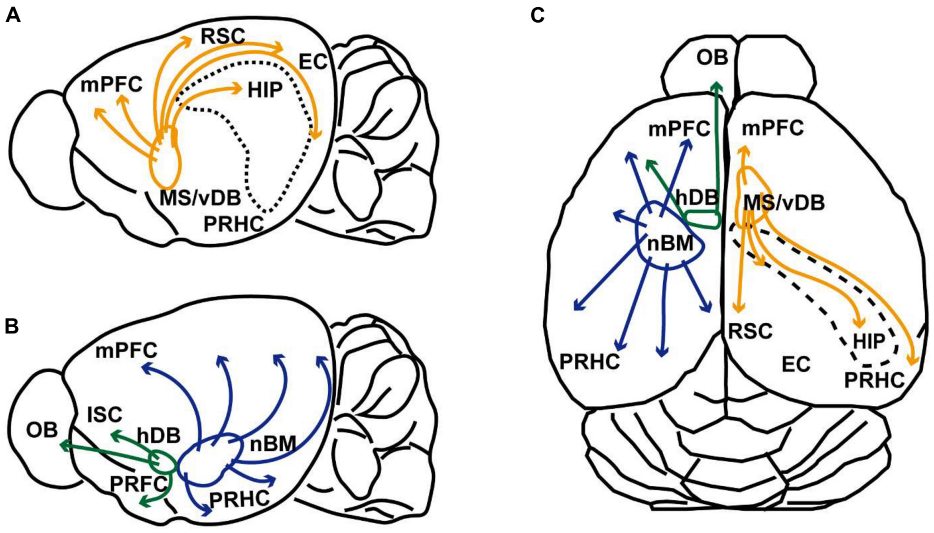

FIGURE 1
Schematic illustrations of cholinergic innervation from the basal forebrain of rodent. **(A)** Schematic sagittal view of the rodent brain illustrating cholinergic projection from the medial septum and ventral diagonal band of Broca (MS/vDB) to the medial prefrontal cortex (mPFC), retrosplenial cortex (RSC), entorhinal cortex (EC), hippocampus (HIP, and perirhinal/postrhinal cortices (PRHC). Cholinergic projections are indicated by orange lines. **(B)** Schematic sagittal view of the rodent brain showing cholinergic projection from the horizontal diagonal band of Broca (hDB) and nucleus basalis magnocellularis (nBM). Cholinergic neurons in the hDB innervates the olfactory bulb (OB), insular cortex (ISC) and piriform cortex (PRFC). Cholinergic neurons in the nBM project to the entire cortex including the mPFC and PRHC. Cholinergic modulations are indicated green and blue lines. Projections to the amygdala are omitted from the illustration. **(C)** Schematic dorsal view of the rodent cholinergic system. The right hemisphere shows cholinergic innervation from the MS/vDB. The left hemisphere indicates cholinergic projections from the hDB and nBM.

Selective cholinergic cell elimination in the MS/vDB by the immunotoxin-mediated cell targeting technique also impairs the object location memory in both multiple-trial and one-trial object recognition memory tasks (Okada et al., 2015; **Figures 2A–C**). One-trial recognition memory task simply consists of a sample trial and a test trial (Ennaceur and Delacour, 1988; Dere et al., 2006), whereas multiple-trial object recognition task is composed of some repeated sample and test trials (Poucet, 1989; Save et al., 1992; Okada et al., 2015). Amount of familiarization in the sample phase is reported to affect the performance in the test trials in object recognition memory (Albasser et al., 2009; Broadbent et al., 2010; Antunes and Biala, 2012). In contrast, another study reported that 192 IgG-saporin cholinergic lesions in the MS do not cause impairment of object location memory (Dashniani et al., 2015), although the difference in behavioral phenotypes may be because of their lesion sizes or subsections. For example, lesion of the MS left approximately 70% cholinergic neurons in the study of Dashniani et al. (2015), and their lesion size seems to be smaller than that in Okada et al. (2015). The injection sites of Dashniani et al. (2015) are located posterior in the MS to the sites of Cai et al. (2012). Injection sites of Okada et al. (2015) included a wide range of the MS/vDB along with the anteroposterior and mediolateral axes. The MS has a clear mediolateral topographical arrangement (Gaykema et al., 1990). The medial part of the MS projects to the dorsal hippocampus, the subiculum, and the lateral entorhinal cortex, whereas the lateral MS mainly projects to the ventral hippocampus, the subiculum, and the medial entorhinal cortex (Gaykema et al., 1990). In addition, neurons in the MS and rostral vDB mainly innervate the entire hippocampus, the subiculum and the entorhinal cortex, while neurons in the caudal vDB projects to the dorsal hippocampus, the dorsal subiculum and the lateral entorhinal cortex (Gaykema et al., 1990). The dorsal and ventral hippocampal structures are differently involved in mnemonic function (Hughes, 1965; Hock and Bunsey, 1998; Moser and Moser, 1998; Cassel et al., 2002). The medial and lateral entorhinal cortices are also differently implemented in the object recognition memory (Aggleton and Nelson, 2020). These anatomical and functional findings suggest that cholinergic neurons in subsections of the MS/vDB are differently involved in object location recognition memory or object-in-place recognition memory.

Cholinergic hippocampal activity is also reported to be important in object recognition memory (Aloisi et al., 1997; Giovannini et al., 2001; Stanley et al., 2012; Rashid and Ahmed, 2019). Neurochemical analysis shows that acetylcholine efflux in the hippocampus increases during spatial novelty and object exploration (Aloisi et al., 1997; Giovannini et al., 2001; Stanley et al., 2012). Pharmacological studies also indicate that cholinergic activity in the hippocampus and parahippocampal areas plays a role in novelty preference

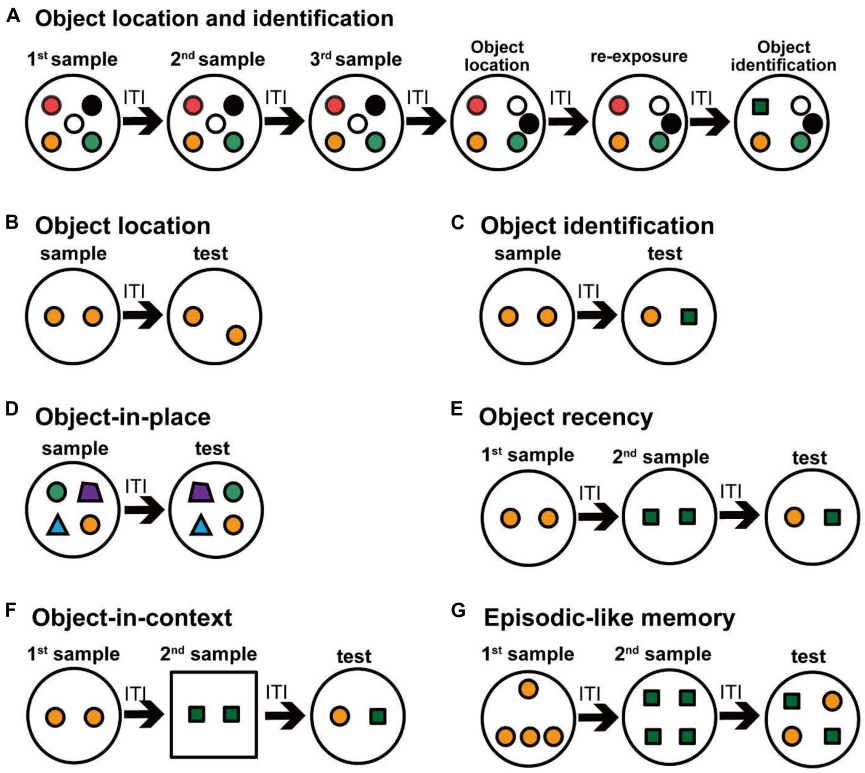

FIGURE 2
Schematic drawing of various object recognition tasks in rodents. Small colored circles and polygons indicate objects in an open field. Experimental protocols for evaluating the object recognition memory are shown. **(A)** The multiple-trial task evaluates the object recognition memory for the location and identification of the objects. In this task, successive six exposures are conducted with an ITI within 1 day. After three trials of sample exposure, two objects were relocated and an object location test is conducted. After re-exposure to the same arrangement objects in the object location test, a familiar object is replaced by a novel object in the object identification test. **(B–G)** One-trial tasks evaluate the object recognition memory, in which a sample trial and a test trial are conducted with an ITI on the same day, and some changes in the experimental conditions as for the objects are made in the test trial. In the object location task **(B)**, one of two objects is relocated in the test trial. In the object identification task **(C)**, one of two objects is replaced with another object in the test trial. In the object-in-place task **(D)**, two of four objects are relocated in the test trial. In the object recency task **(E)**, two objects in the first sample are exchanged by two other objects in the second sample, and then different objects in two samples are presented in the test trial. In the object-in-context task **(F)**, a set of objects in a context in the first sample are replaced with another set of objects in a different context in the second sample, and then different objects in two samples in the first context are presented in the test trial. In the episodic-like memory task **(G)**, four objects in the first samples are exchanged by four other objects in the second sample, and then the objects consisted of two objects from each sample are presented in the test trial.

in several types of object recognition memory task. For example, the activity of muscarinic acetylcholine receptors in the hippocampus and entorhinal cortex is involved in the acquisition and retrieval of object location memory (Rashid and Ahmed, 2019). Acute activation of nicotinic receptors in the hippocampus or perirhinal cortex similarly enhances the acquisition of object recognition memory and object location memory, but not the retrieval of these memories (Melichercik et al., 2012). Local scopolamine infusion indicates that muscarinic activity in the hippocampus and perirhinal cortex is involved in short-term (90 min) object recognition memory, but muscarinic activity in the perirhinal cortex plays a role also in long-term (24 h) object recognition memory (Balderas et al., 2012). These results suggest that cholinergic hippocampal activity is involved in the performance of object location memory. It is unknown how cholinergic hippocampal activity modulates object recognition memory.

Cholinergic lesions of the MS with 192 IgG-saporin cause a deficit in object-in-context recognition memory, but not in episodic-like object recognition memory (Easton et al., 2011; **Figures 2F,G**). This impairment in object-in-context memory is suggested to be caused by failure in rapid updating of place cells when the object changes its environment. Indeed, MS cholinergic lesions with 192 IgG-saporin impair the development of new place cell representation in a novel context (Ikonen et al., 2002). Scopolamine infusion alters the firing properties of hippocampal place cells and grid cells in the entorhinal cortex (Brazhnik et al., 2004; Newman et al., 2014). Exploration in novel environments influences the firing properties of place cells and grid cells, suggesting that the increase of acetylcholine release in novel environment is related

to alternation of firing patterns of these cells (Barry et al., 2012). Therefore, cholinergic activity in the hippocampus is strongly related to memory with salient spatial components.

Cholinergic projections from the nucleus basalis of Meynert

Previous studies have revealed that cholinergic neurons in the nBM are important in a different type of object recognition memory from cholinergic neurons in the MS/vDB. A cholinergic lesion in the nBM by 192 IgG-saporin does not cause a novelty preference deficit in the object recognition memory test after 60-min delay (Savage et al., 2011). A selective cholinergic ablation in the nBM by the immunotoxin-mediated cell targeting technique also shows an intact novelty preference in the multiple-trial object recognition memory task, but it causes the impairment in one-trial object recognition memory after 3–30-min delays (Okada et al., 2015; **Figures 2A,C**).

Cholinergic neurons in the nBM project to the neocortex and amygdala, but also to the frontal, entorhinal, and perirhinal cortices (Woolf and Butcher, 1982, 1985; Rye et al., 1984; Woolf et al., 1984; Carlsen et al., 1985). Cholinergic transmission in the perirhinal cortex is reported to play a pivotal role in object recognition memory (Brown et al., 2012). Local infusion of methyllycaconitine or scopolamine in the perirhinal cortex impairs the acquisition of object recognition memory (Abe and Iwasaki, 2001; Winters and Bussey, 2005; Tinsley et al., 2011). Acute and pre-sample nicotinic receptor activation in the perirhinal cortex enhances novelty preference in the object recognition memory task (Melichercik et al., 2012). On the other hand, the cholinergic activity in the perirhinal cortex is not necessary for the retrieval of object recognition memory. Local scopolamine infusion into the perirhinal cortex does not affect object recognition memory during the test trial (Winters et al., 2006). Moreover, cholinergic activity in the perirhinal cortex is important in other variations of object recognition memory such as object-in-place and object recency memory (Brown et al., 2012; **Figures 2D,E**). Some studies have reported that the perirhinal cortex has no role in the object recognition memory in the absence of visual information (Winters and Reid, 2010; Albasser et al., 2013).

Acetylcholine in the medial prefrontal cortex is involved in novelty preference in the object recognition memory task (Esaki et al., 2021a,b). Nicotinic activation in the medial prefrontal cortex enhances the performance of object recognition memory (Esaki et al., 2021a,b). Scopolamine infusion into the medial prefrontal cortex impairs the acquisition of object-in-place recognition memory, but not the retrieval of the memory (Esaki et al., 2021a,b). This treatment also impairs the object recency memory (Barker and Warburton, 2011). Acetylcholine release in the prefrontal cortex is necessary for attention (Dalley et al., 2004; Nyberg, 2005; Bloem et al., 2014), suggesting that cortical cholinergic activity might be related to the acquisition of object recognition memory through its novelty-induced attention.

Cholinergic projections from the horizontal diagonal band of Broca

There seems to be no report which indicates that cholinergic neurons of the hDB are related to object recognition memory, though cholinergic lesions in this area have been reported to increase depressive-like behaviors (Chen et al., 2021). The piriform cortex is reported to be important in processing odor-object recognition and integrating multisensory object information (Porada et al., 2019). On the other hand, there is the possibility that cholinergic projection to the perirhinal cortex is involved in object recognition memory *via* the hDB (Winters and Bussey, 2005). It is an issue to be addressed whether cholinergic projection from the hDB to the piriform and perirhinal cortices play a role in the processing of object recognition memory.

Cholinergic interneurons

Striatal cholinergic interneurons are regarded as tonically active neurons (Kimura, 1986; Inokawa et al., 2010), and modulate striatal dopaminergic activity (Calabresi et al., 2000; Wang et al., 2006). Striatal cholinergic interneurons play a role in cognitive processes such as spatial working memory, reward-related learning (Kitabatake et al., 2003), habit learning (Packard and Knowlton, 2002; Aoki et al., 2018; Amaya and Smith, 2021), and behavioral flexibility (Ragozzino et al., 2009; Okada et al., 2014; Prado et al., 2017). Mice deficient in the vesicular acetylcholine transporter in the striatum have been reported to show impairment in short-term (15-min delay) object recognition memory (Palmer et al., 2016), indicating that cholinergic activity in the striatum is also relevant to the acquisition of object recognition memory. In contrast, there have been no reports to date on the role of cortical cholinergic interneurons in object recognition memory.

Behavioral factors affecting object recognition memory

In the object recognition task, the experimenter uses the rodents' inherent behavioral treat with their exploration and preference to the novelty, in order to evaluate the animals' recognition memory. The rodents are able to react and re-explore the objects when the objects are altered with various properties, including material, size, and topographical arrangement or location (Cheal, 1978; Sutherland et al., 1982;

Poucet et al., 1986; Thinus-Blanc et al., 1987; Ennaceur and Delacour, 1988; Save et al., 1992). This task does not require learning associated with any rules or any apparent reinforcements, but it is based on the inherent and spontaneous exploratory behavior toward novel or changed objects (Ennaceur and Delacour, 1988). Since the object recognition task uses the rodents' spontaneous novelty preference that is measured by exploration to unfamiliar objects against more familiar objects, it is inevitable that the mentioned behavioral parameters of exploratory activity and attention would interfere the estimation of the object recognition memory (Antunes and Biala, 2012).

Exploration in the open field

Evaluation of object recognition memory is based on the comparison between the explorations to unfamiliar and familiar objects in the test phase. When the animals show the lack or deficit of exploratory behavior itself, they are excluded from the data analysis of the experiments (Ennaceur and Delacour, 1988; Tinsley et al., 2011). Microdialysis studies in rodents have demonstrated that acetylcholine release in the cortex and hippocampus increases during exploration in a novel open field (Aloisi et al., 1997; Thiel et al., 1998; Giovannini et al., 2001). This increment of the acetylcholine levels gets shorter and smaller during re-exposure to the open field, suggesting that cholinergic activity is associated with exploration for novelty and declines according to habituation (Giovannini et al., 2001).

Cholinergic lesions in the basal forebrain by 192 IgG-saporin and systemic scopolamine administration do not alter rodents' behavior in the open field (Psyrdellis et al., 2016; Dobryakova et al., 2018). In contrast, another report showed that cholinergic lesions led to hyperactivity in the open field (Waite et al., 1995). Systemic high-dose treatment (> 0.03 mg/kg) of scopolamine has been reported to impair locomotor activity (Klinkenberg and Blokland, 2010). These contradictory results suggest that the locomotor activity during the exploration appears to be altered by cholinergic dysfunction, depending on differences in the severity and location of the cholinergic lesion.

Seeking novelty and attention

Animals show the novelty preference dependent on the integrity of their attention and memory in the test phase of object recognition memory (Silvers et al., 2007; Antunes and Biala, 2012). Several studies have shown that novelty signals during learning are associated with hippocampal or cortical acetylcholine transmission (Wilson and Rolls, 1990; Hasselmo, 1999; Ranganath and Rainer, 2003; Meeter et al., 2004; Barry et al., 2012). Acute nicotine administration improves attention and memory (Levin et al., 2006), and enhances novelty detection and subsequent recognition memory (Froeliger et al., 2009). Administration of scopolamine and mecamylamine revealed that nicotinic and muscarinic receptors are also important in attentional processing (Mirza and Stolerman, 1998, 2000; Klinkenberg and Blokland, 2010). A selective cholinergic lesion of the nBM or prefrontal cortex impairs attention and visual cue detection (McGaughy and Sarter, 1998; McGaughy et al., 2002; Chudasama et al., 2004; Klinkenberg and Blokland, 2010), suggesting that cholinergic modulation of attention and cue detection is mediated by the prefrontal cortex. The basal forebrain cholinergic system appears to regulate object recognition memory, at least partly, through attention.

Impairments in object recognition memory in animal models for Alzheimer's disease

Alzheimer's disease is a progressive dementia. This disease is characterized by anterograde amnesia of short-term episodic memory, together with impairment in attention and spatial recognition at the early stage (Snowden et al., 2011). Impairment in recognition memory frequently occurs in patients at the prodromal stage of cognitive symptoms (Ally, 2012), and recognition memory deficit is one of biomarkers of Alzheimer's disease (Russo et al., 2017; Goldstein et al., 2019). Cholinergic neurons in the basal forebrain are highly vulnerable to the effects of tauopathy in Alzheimer's disease, and neuronal loss is generated in the basal forebrain area, but cholinergic cell loss is more severe in the nBM than in the MS/vDB (Geula et al., 2021). To mimic the key components associated with the early stage of Alzheimer's disease, a selective elimination of cholinergic neurons in the rodent basal forebrain has been conducted for use as a valid model of Alzheimer's disease at the early stage (Cutuli et al., 2009, 2013; Okada et al., 2015). These model mice show alterations in object recognition memory and object location memory (Cutuli et al., 2013; Okada et al., 2015).

Alzheimer's disease is characterized by neuronal degeneration with the extracellular amyloid plaques and intracellular neurofibrillary tangles (Murphy and LeVine, 2010). The amyloid plaques are composed mainly of amyloid beta (Aβ) derived from the processing of amyloid precursor protein (APP), and neurofibrillary tangles are formed by hyper-phosphorylated tau protein (Zhang et al., 2006; Schmidt et al., 2009; De Strooper, 2010; Murphy and LeVine, 2010). Transgenic mouse models with some mutations in the genes encoding APP, presenilin, and tau have been reported to show deficits in object recognition memory (Dodart et al., 2000; Huang et al., 2006; Middei et al., 2006; Hillen et al., 2010; Zhang et al., 2012; Spilman et al., 2014; Grayson et al., 2015; Mehla et al., 2019). Moreover, object recognition memory was impaired by the intracerebroventricular injection of Aβ (Tsunekawa et al., 2008;

Meunier et al., 2013). Deficits of object recognition memory in these model mice were rescued by the treatment of donepezil as an acetylcholinesterase inhibitor (Zhang et al., 2012), although there are contradictory results in other studies (Tsunekawa et al., 2008; Spilman et al., 2014). The impairments in object recognition memory and object location memory in themodels with cholinergic deletions have been reported to be recovered by treatment with donepezil or rivastigmine (Cutuli et al., 2013; Okada et al., 2015). Although it is still unknown how cholinergic activity is related to the neuropathology and cognitive decline, the object recognition memory task is a useful tool to study the mechanisms underlying the pathology of Alzheimer's disease, and develop therapeutic treatments for dementia.

Future aspects

This review revealed that distinct cholinergic cell groups in the basal forebrain are related to different types of object recognition memory. Cholinergic neurons in the MS/vDB innervating the hippocampal area are involved in object location recognition memory. Cholinergic neurons in the nBM projecting mainly to the entire neocortex have a role in object recognition memory. The perirhinal cortex plays an important role in object recognition memory, and receives cholinergic innervation from both the MS/vDB and nBM. Cholinergic activity in the prefrontal cortex is also necessary for object recognition memory. It is needed to determine which cholinergic cell groups projecting to the perirhinal or prefrontal cortex contribute to object recognition memory. Moreover, the contribution of cholinergic interneurons in the striatum and neocortex remains unknown. In addition, deficits in recognition memory are replicated in various rodent models of several neurological disorders, and the deficits can be rescued by cholinesterase inhibitors that activate cholinergic activity. It is unknown how the inhibitors work for the recovery of mnemonic dysfunctions caused by the neuronal degeneration in Alzheimer's disease. Further experiments will help to explain how the distinct cholinergic neurons could control the cholinergic projection areas during the processes of object recognition memory. Elucidating the cholinergic regulation of object recognition memory will be useful for the development of therapeutic treatments for dementia.

Author contributions

KO: writing—original draft, review, and editing and drawing illustrations. KH: writing—review and editing. KK: writing—original draft, review, and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A critical role for long-term potentiation mechanisms in the maintenance of object recognition memory in perirhinal cortex revealed by the infusion of zeta inhibitory pseudosubstrate

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Object recognition, the ability to discriminate between a novel and a familiar stimulus, is critically dependent upon the perirhinal cortex. Neural response reductions upon repetition of a stimulus, have been hypothesized to be the mechanism within perirhinal cortex that supports recognition memory function. Thus, investigations into the mechanisms of long-term depression (LTD) in perirhinal cortex has provided insight into the mechanism of object recognition memory formation, but the contribution of long-term potentiation (LTP) to object recognition memory formation has been less studied. Inhibition of atypical PKC activity by Zeta Inhibitory Pseudosubstrate (ZIP) impairs the maintenance of LTP but not LTD, thus here infusion of ZIP into the perirhinal cortex allowed us to investigate the contribution of LTP-like mechanisms to object recognition memory maintenance. Infusion of ZIP into the perirhinal cortex of rats 24 h after the sample phase impaired performance in an object recognition but not an object location task, in contrast infusion of ZIP into the hippocampus impaired performance in an object location but not an object recognition task. The impairment in object recognition by ZIP was prevented by administration of the peptide GluA2$_{3y}$, which blocks the endocytosis of GluA2 containing AMPA receptors. Finally, performance in a perceptual oddity task, which requires perirhinal cortex function, was not disrupted by ZIP. Together these results demonstrate the importance of LTP-like mechanisms to the maintenance of object recognition memory in the perirhinal cortex.

KEYWORDS

novel object recognition memory, perirhinal cortex, long-term potentiation, ZIP, rat

Introduction

The perirhinal cortex (PRH) is necessary for object recognition memory and is also a storage site for such memory [for review Warburton and Brown (2010), Brown et al. (2012), and Brown and Banks (2015)]. Critically, PRH interventions that target plasticity mechanisms while leaving neurotransmission intact also impair single item object recognition memory (Warburton et al., 2005; Griffiths et al., 2008; Tinsley et al., 2012). To date, the evidence strongly indicates the involvement of plasticity mechanisms that result in synaptic weakening and have parallels in processes underlying long-term depression (LTD) (Warburton et al., 2003; Griffiths et al., 2008). A process involving synaptic weakening readily explains the observed reduction in neuronal responses observed in monkeys and rats when novel stimuli are seen again (Zhu et al., 1995; Xiang and Brown, 1998) and is consistent with computational modeling predictions that efficient storage in a familiarity discrimination network is only achievable if the learning algorithm includes a term producing synaptic weakening (Bogacz and Brown, 2003). However, an efficient network must maintain a balance of synaptic excitability to avoid either over- or under-reactivity. The question therefore arises as to whether recognition memory also relies on synaptic strengthening within PRH and, if so, does this strengthening process employ long-term potentiation-like mechanisms (LTP).

The atypical protein kinase C isoforms (protein kinase Mζ and ι/λ isoforms) are necessary for the maintenance of LTP but not LTD in the PRH and hippocampus (HPC) (Ling et al., 2002; Sajikumar et al., 2005; Serrano et al., 2005; Panaccione et al., 2013). Application of Zeta Inhibitory Pseudosubstrate (ZIP), an inhibitor of protein kinase Mζ (PKMζ) reversed a previously established LTP (Sajikumar et al., 2005) but not LTD and erased a spatial memory (Pastalkova et al., 2006). While the specificity of ZIP for PKMζ has been questioned (Wu-Zhang et al., 2012; Lee et al., 2013; Volk et al., 2013; LeBlancq et al., 2016) and at higher doses ZIP can act on PKCλ (Ren et al., 2013), the ability of ZIP to impair LTP but not LTD has not been disputed and the effect of ZIP administration on PRH memory function has not been studied. Atypical PKCs are thought to maintain memories by sustaining enhanced AMPA receptor levels in the post-synaptic density *via* an interaction with the GluA2 subunit of the AMPA receptor. The synthetic peptide $GluA2_{3y}$ mimics the carboxy tail of the AMPA receptor and inhibits GluA2 receptor endocytosis. Thus, infusion of $GluA2_{3y}$ prevented the amnesic effects of ZIP infusion in the HPC (Migues et al., 2010), amygdala (Migues et al., 2010), and medial prefrontal cortex (Evuarherhe et al., 2014).

The present study tested the following hypotheses 1. That ZIP infusion into PRH impairs the maintenance of single item novel object recognition but not object location memory 2. As object location but no single item novel object recognition depends on the HPC, we predict the reverse to be true in HPC. 3. ZIP infusion does not alter the perceptual functions of PRH. 4. That the memory impairment produced by ZIP infusion can be blocked by preventing GluA2 receptor endocytosis by infusion of the synthetic peptide $GluA2_{3y}$.

Methods

Subjects

All experiments were conducted on adult male Dark Agouti rats (Bantin and Kingman, Hull, United Kingdom) weighing 230–250 g at the commencement of experiments. Animals were housed in pairs under a 12 h light/dark cycle (light phase, 20.00–08.00.). Behavioral training and testing were conducted during the dark phase of the cycle. Food and water were available *ad libitum*. All animal procedures were performed in accordance with United Kingdom Animals Scientific Procedures Act (1986). All efforts were made to minimize the suffering and the number of animals used.

Four cohorts of rats were used in this study. Cohort 1 consisted of 12 animals and was used to test the effect of ZIP infusion into PRH on the maintenance of object recognition and object location memory, two animals were lost from this cohort due to cannula blockages. Cohort 2 consisted of 10 animals and was used to test the effect of ZIP infusion into HPC on the maintenance of object recognition and object location memory, one animal was lost due to a blocked cannula. These animals had previously received infusion of D-AP5, data reported in Barker and Warburton (2015). Cohort 3 consisted of 13 animals and was used to test the effect of ZIP infusion into PRH on perceptual function, one animal was lost due a blocked cannula. Cohort 4 consisted of 12 animals and was used to test the effect of $GluA2_{3y}$ infusion on ZIP-induced memory impairments, two animals were lost from this cohort due to blocked cannula.

Cannula implantation

Implantation of cannulae followed previously described procedures (Warburton et al., 2003; Barker and Warburton, 2015). Briefly each rat was anesthetized with isoflurane (induction 4%, maintenance 2–3%) and secured in a stereotaxic frame with the incisor bar set to achieve flat skull. Stainless steel guide cannulae (26 gauge, Plastics One, Bilaney, Sevenoaks, United Kingdom) were implanted through burr holes in the skull at the following coordinates relative to bregma: HPC AP −4.8 mm, ML ± 2.6 mm, DV −3.0 mm from dura matter; PRH AP −5.6 mm, ML ± 4.5 mm, DV −6.7 mm from skull surface at 20° to vertical. All cannulae were anchored to the skull by stainless steel screws (Plastics One, Bilaney, United Kingdom) and dental acrylic. Following surgery, each animal received fluid replacement (5 mL saline, s.c.) and analgesia (0.05 mL Temgesic,

i.m.) and then housed individually for 1-week post-surgery and then in pairs. Between infusions, 33-gauge obturators (Plastics One, Bilaney, United Kingdom) kept the cannulae patent.

Intracerebral infusions

The selective PKMzeta inhibitor, ZIP (Tocris, Bristol, United Kingdom) or a scrambled ZIP peptide control (sZIP) (Tocris, Bristol, United Kingdom) were dissolved to a concentration of 10 mM (Pastalkova et al., 2006; Serrano et al., 2008) in physiological saline. The inhibitor of activity dependent endocytosis of GluA2 ($GluA2_{3y}$) was conjugated to the HIV viral transduction domain (TAT) to allow the peptide to penetrate neuronal cell membranes. TAT-$GluA2_{3y}$ (Anaspec, Fremont, USA) or scrambled TAT-$GluA2_{3y}$ (Anaspec, United States) were dissolved to a concentration of 30 μM (Migues et al., 2010) in physiological saline. Infusions followed previously described procedures (Warburton et al., 2003), briefly, infusions were made through a 33-gauge infusion needle (Plastics One, Bilaney, United Kingdom) inserted into the implanted cannulae and attached to a 25-μL Hamilton syringe via polyethylene tubing. Drugs were infused into the HPC at a rate of 0.25 μl min^{-1} and into the PRH at a rate of 0.5 μl min^{-1} over a period of 2 min. The volumes have been used extensively previously (Winters and Bussey, 2005; Akirav and Maroun, 2006; Barker and Warburton, 2008) and have been shown to achieve a drug spread of 1–1.5 mm^3 (Martin, 1991; Attwell et al., 2001). Following the infusion, the needle remained in place for a further 5 min.

To test the effects of ZIP on memory maintenance, ZIP or sZIP was infused 24 h after the sample phase, and memory was tested at a delay of 48 h (**Figure 1B**). This timing has been used previously (Pastalkova et al., 2006; Migues et al., 2010) as it allows information to be encoded and for memory to undergo consolidation before ZIP infusion and allows sufficient time after ZIP infusion for memory retrieval not to be affected by the infusion procedure. $GluR2_{3y}$ was infused 1 h before ZIP infusion as this timing has previously been demonstrated to prevent ZIP induced amnesia (Migues et al., 2010). To test the effects of acute ZIP infusion on PRH function in the perceptual oddity task ZIP was infused 15 min before the task. This delay between infusion and behavior is routinely used to test drug effects on PRH function [for example see Barker et al. (2006) and Barker and Warburton (2008, 2015)]. Experiments were performed using a within-subject cross-over design, thus each animal received both a drug and vehicle infusion in each experiment, with a minimum 48 h gap between each infusion.

Histology

At the completion of the study each rat was anesthetized with Euthetal (Rhone Merieux, Lyon, France) and perfused transcardially with phosphate buffered saline followed by 4% paraformaldehyde. Following removal, the brain was postfixed in paraformaldehyde for a minimum of 2 h then transferred to 30% sucrose in 0.2 M phosphate buffer for 48 h. Coronal sections (50 μm) were cut on a cryostat and stained with cresyl violet. Cannulae locations were checked against a rat brain atlas (Swanson, 1998).

Behavioral testing

Apparatus

Exploration occurred in an open-topped arena 1 m^2 made of wood, with sawdust on the floor. The walls inside the arena were surrounded with a black cloth to a height of 1.5 m to obscure external visual stimuli (the black cloth was removed for the object location task). An overhead camera and a video recorder recorded the animal's behavior for subsequent analysis. The stimuli presented were copies of objects composed of "Duplo" (Lego United Kingdom, Slough, United Kingdom) that varied in shape, color, and size and were too heavy for the animal to displace.

Pretraining

After being handled for 1 week, the animals were habituated to the empty arena for 5 min daily for 4 days before the commencement of the behavioral testing.

Single item novel object recognition memory

The NOR task (**Figure 1A**) comprised a sample phase, followed by an object preference test after a delay of 48 h. In the sample phase, duplicate copies of an object were placed near the two corners at either end of one side of the arena (15 cm from each adjacent wall). The animal was placed into the arena facing the center of the opposite wall and allowed a total of either 40 s of object exploration or 4 min in the arena. At test (3 min duration), the animal was replaced in the arena, presented with two objects in the same positions: one object was a third copy of the set of the objects used in the sample phase, and the other object was a novel object. The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals.

Object location task

This task comprised a sample phase and a test phase separated by a 48 h delay (**Figure 1B**). In the sample phase (4 min duration), the subjects were presented with two identical objects placed near the two corners at either end of one side of the arena and the amount of exploration of each object was recorded by the experimenter. In the test phase (3 min duration) another identical copy of the

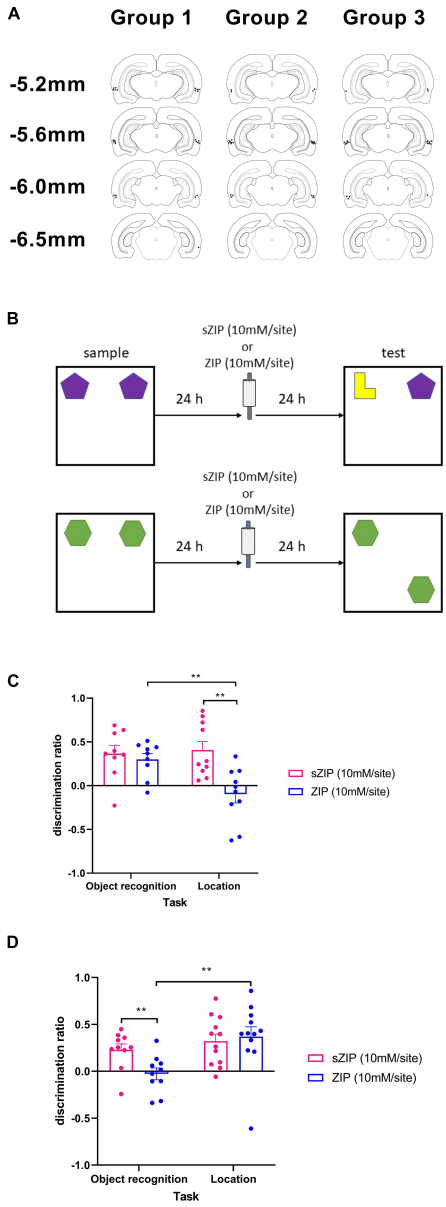

FIGURE 1
Infusion of ZIP into PRH impaired the maintenance of object recognition but not object location memory. **(A)** Location of cannulae tips targeting PRH in the three groups of animals used in this study. **(B)** Outline of novel object recognition and object location tasks used, ZIP was infused 24 h after the sample phase and 24 h before the test phase. **(C)** Performance in the object location but not the object recognition task was significantly impaired following infusion of ZIP into the HPC. **(D)** Performance in the object recognition task but not the object location task was significantly impaired following infusion of ZIP into PRH. Data presented as mean + sem, $^{**}p < 0.01$, HPC: NOR $n = 9$, OL $n = 10$, PRH: NOR $n = 10$, OL $n = 12$.

object was placed in the same position as during the sample phase, while a fourth identical object was placed in a novel location. The position of the moved object was counterbalanced between rats.

Simultaneous oddity discrimination task

In the perceptual oddity task three objects were presented to the rat simultaneously in a line in the center of the arena (Figure 2A). Two objects were identical, while one object was visually different. Each subject was allowed to explore these three objects for a total of 5 min. In a rat where perception is unimpaired, it has been observed that the animal will spend more time exploring the different object compared to the two identical objects (Bartko et al., 2007). This task was first carried out using a pair of objects with low feature ambiguity. Low feature ambiguity objects are pairs of objects that have few visually overlapping features and are therefore considered less perceptually challenging for the rat to discriminate between (Figure 2B). The task was made more perceptually difficult by repeating it with a pair of objects that had greater feature overlap (Figure 2C). The PRH has been shown to be critical in perceptual discrimination when the stimuli to be discriminated have a high degree of feature overlap (Bussey et al., 2002; Bartko et al., 2007), therefore if ZIP infusion is disrupting PRH function animals' performance will be impaired in the high feature ambiguity condition.

Data analysis

All measures of exploration were made with the experimenter blind to the drug status of each animal. Exploratory behavior was defined as the animal directing its nose toward the object at a distance of <2 cm. Any other behavior, such as looking around while sitting on or resting against the object, was not considered as exploration. Discrimination between the objects was calculated using a discrimination ratio (DR), calculated as the absolute difference in the time spent exploring the novel and familiar objects divided by the total time spent exploring the objects. In the simultaneous oddity discrimination task a preference index was calculated as the time spent exploring the 'different' divided by the time spent exploring all three objects. Group comparisons used ANOVA and additional analyses examined whether individual groups had discriminated between the objects, using a one-sample *t*-test (two-tailed) against chance performance (0 for object recognition and location, 0.33 for the oddity discrimination task). All statistical analyses used a significance level of 0.05.

Results

Histology

Histological examination the PRH group confirmed that the cannulae tips were located in the PRH between AP −5.2 mm and AP −6.3 mm relative to bregma (Figure 1A) and in the HPC

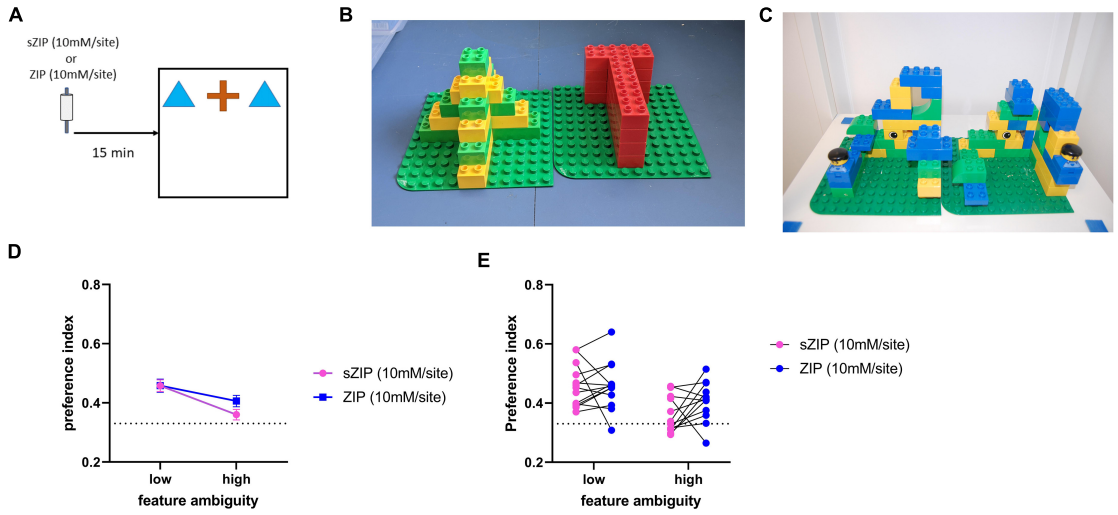

FIGURE 2
Performance in the perceptual oddity task is not altered by ZIP infusion into the perirhinal cortex. **(A)** Outline of the perceptual oddity task used, ZIP was infused into PRH 15 min before the task commenced. **(B)** Example of low and **(C)** high feature ambiguity objects used in the perceptual oddity task. **(D)** Performance in the perceptual oddity task was not altered in either the low or high feature ambiguity condition by infusion of ZIP, dotted line indicates chance performance levels, data presented as mean ± sem. **(E)** Performance of each individual animal after infusion of sZIP or ZIP on both high and low feature ambiguity conditions. Dotted line represents chance performance (0.33). Low feature ambiguity n = 13 for both sZIP & ZIP, high feature ambiguity n = 12 for both sZIP and ZIP.

group all cannulae tips were located in the HPC between the dorsal CA1 and CA3 subfields [see **Figure 2A** and Barker and Warburton (2015) for cannula locations].

Infusion of zeta inhibitory pseudosubstrate into hippocampus selectively impaired the maintenance of object location memory while infusion of zeta inhibitory pseudosubstrate into perirhinal cortex selectively impaired the maintenance of novel object recognition memory

To examine the effect of ZIP infusion into PRH and HPC on the maintenance of object recognition and object location memory, ZIP (10 mM/site) or the scrambled inactive version of the peptide [sZIP (10 mM/site)] was infused 24 h after the sample phase during a 48 h delay between the sample and test phase (**Figure 1B**).

Intra-HPC ZIP significantly impaired object location (OL) performance but had no effect on NOR (**Figure 1C**). Thus, a two-way ANOVA with task and treatment as factors revealed a significant interaction [$F_{(1,17)} = 5.04$, $p = 0.038$] and a significant main effect of treatment [$F_{(1,17)} = 8.56$, $p = 0.009$], but no significant main effect of task [$F_{(1,17)} = 4.34$, $p = 0.053$]. Analysis of the simple main effects revealed that the performance of ZIP infused animals was significantly poorer than the performance of sZIP infused animals in the OL task ($p = 0.008$) and performance in the ZIP infused animals was significantly different between the NOR and OL tasks ($p = 0.005$). Performance of the sZIP infused animals was not significantly different between the two tasks ($p = 0.767$). Further analysis revealed that in the NOR task both sZIP [$t_{(8)} = 3.90$, $p = 0.005$] and ZIP [$t_{(8)} = 4.432$, $p = 0.002$] infused animals showed significant discrimination between the novel and the familiar object, in contrast in OL, sZIP [$t_{(9)} = 4.16$, $p = 0.002$] but not ZIP [$t_{(9)} = -0.99$, $p = 0.350$] infused animals showed significant discrimination between the moved and unmoved objects.

Intra-PRH infusion of ZIP significantly impaired NOR performance but had no effect on OL performance (**Figure 1D**). A two-way ANOVA with treatment and task as factors revealed a significant interaction [$F_{(1,20)} = 5.27$, $p = 0.033$] and a significant main effect of task [$F_{(1,20)} = 6.82$, $p = 0.017$] but no significant main effect of treatment [$F_{(1,20)} = 2.41$, $p = 0.136$]. Analysis of the simple main effects revealed that the performance of ZIP-infused animals was significantly poorer than sZIP-infused animals in the NOR ($p = 0.004$) and the performance of the ZIP infused animals was significantly poorer in the NOR task compared to the OL task ($p = 0.006$). There was no significant difference in the performance of the sZIP infused animals between the two tasks (p = 0.368). Further analysis revealed that in the OL task both sZIP [$t_{(11)} = 4.29$, $p = 0.001$] and ZIP [$t_{(11)} = 3.56$, $p = 0.004$] infused animals showed significant discrimination between the moved and unmoved object, in contrast in the NOR task sZIP [$t_{(9)} = 3.61$, $p = 0.006$] but not ZIP [$t_{(9)} = -0.43$, $p = 0.674$] infused animals showed significant discrimination between the novel and familiar object.

TABLE 1 Mean exploration times in the sample and test phases across all three tasks tested.

Figures	Infusion site	Task	Infusate	Exploration in sample phase (s)	Exploration in test phase (s)
Figure 1C	HPC	NOR.	sZIP	32.1 ± 2.1	23.3 ± 4.2
			ZIP	28.6 ± 2.5	21.8 ± 1.6
		OL.	sZIP	33.3 ± 4.3	16.9 ± 1.5
			ZIP	41.4 ± 4.3	18.4 ± 2.1
Figure 1D	PRH	NOR.	sZIP	22.7 ± 2.0	18.3 ± 1.5
			ZIP	23.3 ± 2.4	19.8 ± 2.0
		OL.	sZIP	33.1 ± 3.7	17.7 ± 1.6
			ZIP	32.3 ± 2.3	18.5 ± 0.9
Figure 2D	PRH	PO low FA	sZIP	n/a	40.7 ± 3.3
			ZIP	n/a	41.9 ± 3.9
		PO high FA	sZIP	n/a	44.1 ± 3.4
			ZIP	n/a	45.6 ± 3.2
Figure 3B	PRH	NOR	$sGluA2_{3y}$	26.2 ± 1.5	21.5 ± 2.2
			$GluA2_{3y}$	25.7 ± 2.2	24.6 ± 1.6
Figure 3C	PRH	NOR	$sGluA2_{3y}$ & ZIP	26.1 ± 2.7	25.1 ± 3.3
			$GluA2_{3y}$ & ZIP	27.4 ± 2.4	24.6 ± 2.4

HPC, hippocampus; PRH, perirhinal cortex; NOR, novel object recognition task; OL, object location task; PO, perceptual oddity; FA, feature ambiguity; Data presented as mean ± sem.

There was no significant effect of intra-PRH, or intra-HPC ZIP infusion on overall object exploration levels (Table 1). Analysis of total object exploration in the sample phase revealed no significant interaction between treatment and task with infusion into either the HPC [$F_{(1,17)} = 1.82$, $p = 0.195$] or PRH [$F_{(1,20)} = 1.01$, $p = 0.327$] and no significant main effect of treatment [HPC $F_{(1,17)} = 0.28$, $p = 0.606$; PRH $F_{(1,20)} = 0.01$, $p = 0.921$], however there was a significant main effect of task with infusion into either region [HPC $F_{(1,17)} = 4.78$, $p = 0.043$; PRH $F_{(1,20)} = 1.01$, $p = 0.327$] which reflected a greater level of overall object exploration in the sample phase of the OL task in both sZIP and ZIP infused animals (Table 1). Analysis of the total object exploration in the test phase revealed no significant interaction between treatment and task following infusion of ZIP into the HPC [$F_{(1,17)} = 0.25$, $p = 0.621$] or PRH [$F_{(1,20)} = 0.55$, $p = 0.812$] and no significant main effect of treatment [HPC $F_{(1,17)} = 0.04$, $p = 0.852$; PRH $F_{(1,20)} = 0.55$, $p = 0.466$]. There was a significant main effect of task following infusion into the HPC [$F_{(1,17)} = 6.48$, $p = 0.021$] but not following infusion into the PRH [$F_{(1,20)} = 0.52$, $p = 0.480$].

Infusion of zeta inhibitory pseudosubstrate into perirhinal cortex does not alter perceptual function

To investigate the possibility that ZIP infusion produced deficits in NOR performance by impairing perceptual function, rats were tested in a simultaneous oddity discrimination task (Bartko et al., 2007), with a high feature ambiguity and a low feature ambiguity condition, sZIP or ZIP was infused into PRH 15 min before the task (Figure 2A).

Intra-PRH ZIP did not significantly alter performance in either the low or high feature ambiguity condition. Figures 2D,E confirmed by no significant treatment by feature interaction [$F_{(1,23)} = 1.64$, $p = 0.214$] and no significant main effect of treatment [$F_{(1,23)} = 1.65$, $p = 0.212$]. There was a significant main effect of feature ambiguity [$F_{(1,23)} = 11.65$, $p = 0.002$], due to the poorer performance in the sZIP and ZIP infused animals in the high feature ambiguity condition. Further analysis revealed that in the low feature ambiguity condition both sZIP [$t_{(13)} = 6.26$, $p = 0.00004$] and ZIP [$t_{(13)} = 5.59$, $p = 0.0001$] infused animals showed a significant preference for exploring the different object, while in the high feature ambiguity condition the ZIP infused animals showed significant discrimination [$t_{(11)} = 3.75$, $p = 0.003$] but sZIP infused animals did not [$t_{(11)} = 1.51$, $p = 0.159$]. Analysis of the total object exploration revealed no significant interaction between treatment and feature ambiguity [$F_{(1,23)} = 0.004$, $p = 0.951$] and no significant main effect of treatment [$F_{(1,23)} = 0.15$, $p = 0.702$], however there was a significant main effect of feature ambiguity [$F_{(1,23)} = 4.78$, $p = 0.039$], due to the higher levels of exploration completed by both sZIP and ZIP infused animals in the high feature ambiguity condition (Table 1).

Blocking GluA2 receptor endocytosis prevents the novel object recognition impairment caused by the infusion of zeta inhibitory pseudosubstrate into perirhinal cortex

Infusion of $GluA2_{3y}$ 24 h after the sample phase during a 48 h delay between the sample and test (Figure 3A) phases

did not significantly alter performance (**Figure 3B**). Thus, one-way ANOVA revealed no significant difference between the sGluA2$_{3y}$ and the GluA2$_{3y}$ infused animals [$F_{(1,9)}$ = 0.02, p = 0.880]. Indeed, animals infused with either sGluA2$_{3y}$ [$t_{(9)}$ = 5.87, p = 0.0002] or GluA2$_{3y}$ [$t_{(9)}$ = 2.75, p = 0.022] showed significant discrimination between the novel and familiar objects. In addition, analysis of total object exploration in the sample [$F_{(1,9)}$ = 0.03, p = 0.872] and test phases [$F_{(1,9)}$ = 1.60, p = 0.238] revealed no significant difference between the sGluA2$_{3y}$ and GluA2$_{3y}$ infused animals (**Table 1**).

Infusion of GluA2$_{3y}$ 1 h before ZIP prevented the ZIP induced impairment in performance in the NOR task (**Figure 3C**). Animals infused with sGluA2$_{3y}$ followed by ZIP showed significantly worse performance in the test phase than animals infused with GluA2$_{3y}$ before ZIP. One-way ANOVA revealed a significant main effect of treatment [$F_{(1,9)}$ = 6.66, p = 0.03]. Further analysis revealed that animals infused with GluA2$_{3y}$, and ZIP showed significant discrimination between the novel and familiar object [$t_{(9)}$ = 4.53, p = 0.001], but animals infused with sGluA2$_{3y}$ and ZIP failed to show significant discrimination [$t_{(9)}$ = 0.42, p = 0.687]. Analysis of the total object exploration in the sample [$F_{(1,9)}$ = 0.20, p = 0.666] and test phases [$F_{(1,9)}$ = 0.02, p = 0.903] revealed no significant difference between the sGluA2$_{3y}$/ZIP and GluA2$_{3y}$/ZIP infused animals (**Table 1**).

Discussion

The findings of the current study are fourfold: (1) Infusion of ZIP into PRH impaired the maintenance of NOR but not OL memory (2) Infusion of ZIP into HPC impaired the maintenance of OL but not NOR, (3) ZIP infusion did not alter PRH dependent visual-tactile perception, (4) The action of ZIP in blocking memory maintenance is prevented when AMPA receptor endocytosis is blocked by GluA2$_{3y}$.

Administration of ZIP into the HPC and PRH produced impairments in distinct forms of memory, replicating reports of double dissociations in the effects of HPC and PRH lesions (Winters et al., 2004; Barker and Warburton, 2011). The impairment in OL memory following ZIP infusion into HPC is in line with the well-established role of HPC in spatial memory and replicates previous studies (Hardt et al., 2010; Migues et al., 2010). The role of the HPC in object recognition memory is complex, as some studies fail to report NOR deficits following lesion of the HPC [see Brown and Banks (2015), Cohen and Stackman (2015), and Chao et al. (2020) for reviews], while others have reported NOR deficits specifically following HPC drug infusions [see Cohen and Stackman (2015) and Chao et al. (2020) for reviews]. Here, the failure of intra dorsal HPC ZIP to alter NOR performance replicates a previous study (Hardt et al., 2010). In contrast another study reported that ZIP infusion into the dorsal, intermediate and ventral hippocampus, NOR performance was significantly impaired compared to controls (Hales et al., 2015) although it should be noted that the ZIP-treated animals were still able to discriminate between the novel and familiar objects. Thus, it appears that while the HPC as a whole may play a role in the maintenance of NOR memory, the dorsal HPC alone does not. In addition, any role of the HPC is not as critical as that of the PRH. That intra-PRH ZIP selectively impaired NOR demonstrates that object recognition memory information is stored in the PRH for at least 24 h, in line with findings from *in vivo* recording studies (Xiang and Brown, 1998) and this finding is replicated by a further study in this issue (Augereau and Hardt) which extends the finding to show that 6 days old object memories are also dependent on PRH.

ZIP has been shown to impair the maintenance of LTP but not LTD *in vitro* (Sajikumar et al., 2005; Panaccione et al., 2013), and it has been hypothesized that PKMζ maintains memories by preventing GluA2 receptor endocytosis (Sacktor, 2011), In this study ZIP induced amnesia was prevented by blocking GluA2 receptor endocytosis, using the synthetic peptide GluA2$_{3y}$ indicating that ZIP impairs the maintenance of memory and LTP by the same mechanism. Thus, this study provides evidence that LTP-like in addition to LTD-like mechanisms within PRH are critical for object recognition memory.

Previous reports have failed to find a clear link between LTP and object recognition memory formation within PRH. Thus, blockade of cannabinoid or NR2A receptors which was found to impair LTP, but not LTD produced no impairment in NOR when infused into the PRH (Massey et al., 2004; Barker et al., 2006; Tamagnini et al., 2013). Although some studies have suggested a correlational link between LTP and PRH dependent object memory (Silingardi et al., 2011), The discrepancy in these findings might reflect the involvement of different forms of LTP, or that different forms of plasticity mediate different stages of memory processing (i.e., memory encoding vs. maintenance).

Although a number of studies have demonstrated a link between LTD-like mechanisms in PRH and object recognition memory (Warburton et al., 2003; Griffiths et al., 2008), the observation that LTP-like processes also pay a role is not unexpected. Mathematical models have demonstrated the importance of strengthening some synapses while others are weakened for efficient network function (Norman, 2010) and if synaptic weakening was the only process occurring within PRH then object recognition memory capacity would be highly limited as synaptic weakening alone would lead to a loss of neuronal responses within PRH. However, investigation of human recognition memory revealed subjects were able to remember 10,000 images with the same accuracy as 100 (Standing, 1973), suggesting that humans have a large capacity for recognition. Understanding the relationship between LTD and LTP-like processes during object recognition memory formation and maintenance will be critical to understanding how PRH is able to support the large capacity of object recognition memory.

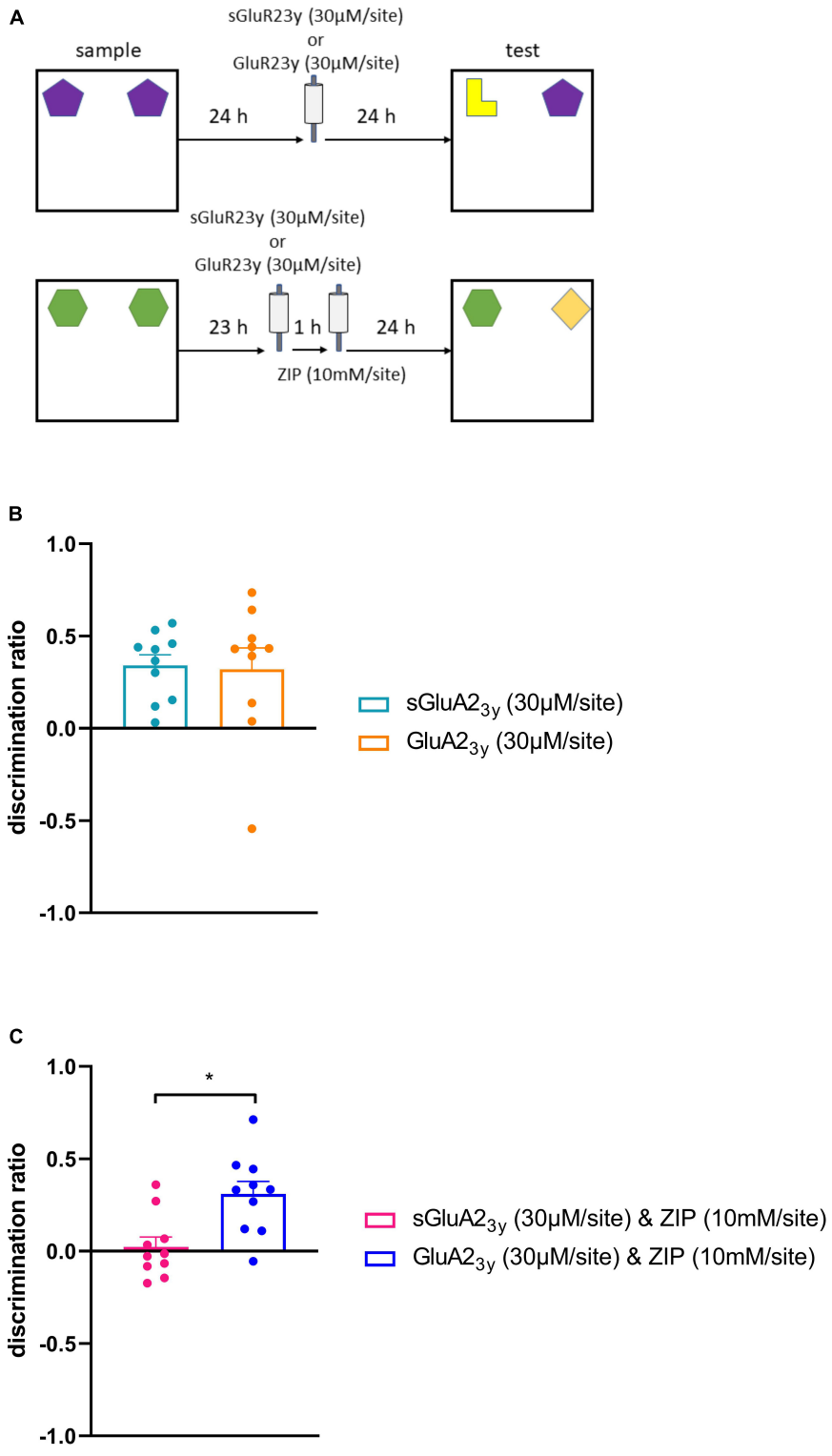

FIGURE 3
Blocking AMPA receptor endocytosis prevents the impairment in object recognition memory maintenance by intra PRH infusion of ZIP. **(A)** Outline of object recognition task and infusion timings, in the first experiment sGluA23y or GluA23y was infused 24 h after the sample phase, in the second experiment infusion of sGluA23y/GluA23y occurred 23 h after the sample phase, 1 h after this infusion ZIP was infused. **(B)** Intra-PRH GluA23y did not alter NOR memory maintenance. **(C)** Infusion of GluA23y into PRH prevented the impairment in NOR maintenance produced by intra-PRH ZIP. Data presented as mean ± sem, all conditions $n = 10$, $^*p < 0.05$.

Infusion of ZIP into either the HPC or PRH did not alter the animals overall object exploration levels in any of the tasks tested in this study, indicating that ZIP did not alter the animals motivation to interact with the stimuli or alter attentional processing. In experiment 1 exploration levels during the sample phase were higher in all conditions during OL compared to NOR, as object exploration in NOR was capped at 40 s, whereas, there was no limit on object exploration in the OL. Given we observed a double dissociation in the effects of HPC and PRH ZIP function on performance in these tasks it is unlikely that this difference in overall exploration levels between the tasks contributed any of the observed deficits.

Zeta inhibitory pseudosubstrate is thought to act by blocking the catalytic domain of atypical PKCs, however a recent report found that infusion of ZIP into the HPC impaired synaptic transmission (LeBlancq et al., 2016). Therefore, to test whether the infusion of ZIP into PRH had a non-specific effect we assessed performance in an oddity discrimination task, in which, when objects have a high degree of feature overlap is sensitive to disruption of synaptic transmission in the PRH (Bussey et al., 2002; Bartko et al., 2007). Here, infusion of ZIP into PRH did not disrupt performance in the perceptual oddity discrimination task, suggesting that ZIP infusion did not disrupt synaptic transmission within PRH. It has been reported that administration of ZIP onto cultured hippocampal neurons can result in cell death (Sadeh et al., 2015), however in the present experiments a within subjects design was used, and animals also received multiple infusions yet no change in behavioral performance was observed following ZIP infusion suggesting that ZIP has not caused large scale cell death. Other studies have also shown that animals can form new memories following ZIP infusion further suggesting that the effect of ZIP is not due to cell death (Pastalkova et al., 2006; Sacktor, 2008; von Kraus et al., 2010; Hales et al., 2015). Therefore, it is unlikely that the observed deficits in performance were due to cell death.

In summary, this study demonstrated that OR memory is maintained in the PRH not the HPC and demonstrated the importance of LTP-like mechanisms within PRH to the maintenance of object recognition memory. Understanding how LTD and LTP like processes interact within PRH will be critical to understanding object recognition memory formation and maintenance.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by Animal Welfare and Ethical Review Body (AWERB), University of Bristol.

Author contributions

AO and GB performed the experiments and analyzed the data. All authors designed the experiments, wrote the manuscript, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Developmental trajectory of episodic-like memory in rats

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Introduction: Episodic memory formation requires the binding of multiple associations to a coherent episodic representation, with rich detail of times, places, and contextual information. During postnatal development, the ability to recall episodic memories emerges later than other types of memory such as object recognition. However, the precise developmental trajectory of episodic memory, from weaning to adulthood has not yet been established in rats. Spontaneous object exploration tasks do not require training, and allow repeated testing of subjects, provided novel objects are used on each trial. Therefore, these tasks are ideally suited for the study of the ontogeny of episodic memory and its constituents (e.g., object, spatial, and contextual memory).

Methods: In the present study, we used four spontaneous short-term object exploration tasks over two days: object (OR), object-context (OCR), object-place (OPR), and object-place-context (OPCR) recognition to characterise the ontogeny of episodic-like memory and its components in three commonly used outbred rat strains (Lister Hooded, Long Evans Hooded, and Sprague Dawley).

Results: In longitudinal studies starting at 3–4 weeks of age, we observed that short term memory for objects was already present at the earliest time point we tested, indicating that it is established before the end of the third week of life (consistent with several other reports). Object-context memory developed during the fifth week of life, while both object-in-place and the episodic-like object-place-context memory developed around the seventh postnatal week. To control for the effects of previous experience in the development of associative memory, we confirmed these developmental trajectories using a cross-sectional protocol.

Discussion: Our work provides robust evidence for different developmental trajectories of recognition memory in rats depending on the content and/or

complexity of the associations and emphasises the utility of spontaneous object exploration tasks to assess the ontogeny of memory systems with high temporal resolution.

KEYWORDS

spontaneous object exploration, object recognition, context, object-place-context, memory ontogeny

Introduction

Episodic memory relies on the coordination of many brain regions that bind multiple memory traces into a coherent spatiotemporal episode (Eichenbaum, 2017). Due to the complexity of neurophysiological processes that underlie it, episodic memory is particularly susceptible to disruptions due to normal ageing, traumatic brain injury as well as virtually all major neurological, neuropsychiatric, neurodevelopmental, and neurodegenerative diseases (Dickerson and Eichenbaum, 2010; Souchay et al., 2013; Vakil et al., 2019). Given the potential important diagnostic and translational value of episodic memory, it is important to study the neural processes that underlie it and its ontogeny.

Understanding when and how episodic memory develops is critical for disentangling the effect of interactions between genetics and experience on the neural circuits and cognitive processes that support it. It is also important for gaining insights into developmental disease progression, and for discovering developmental windows suitable for therapeutic interventions (Guillery-Girard et al., 2013; Souchay et al., 2013; Asiminas et al., 2019). In humans, episodic memory emerges relatively late during juvenile development in comparison to other forms of memory (Gogtay et al., 2004; Guillery-Girard et al., 2013; Mullally and Maguire, 2014; Riggins et al., 2020; Ngo et al., 2021). While children as young as 4 years old are able to retrieve multi-element events, associative memory that is dependent on context discrimination appears to follow a more protracted developmental trajectory (Ngo et al., 2021), which may be connected to late development of prefrontal cortex (Giedd et al., 1999; Gogtay et al., 2004; Eichenbaum, 2017).

The circuitry underlying episodic memory has been studied extensively in rodents, both in the context of basic science as well as a vehicle for understanding the pathophysiology of neurodegenerative and neurodevelopmental disorders (Day et al., 2003; Eacott and Norman, 2004; Good et al., 2007; Langston and Wood, 2009; Davis et al., 2013; Till et al., 2015; Chao et al., 2016, 2020; Asiminas et al., 2019; Barker and Warburton, 2020). A variety of tasks have been used to assess neural mechanisms of episodic memory, including both spontaneous exploration tasks (Eacott and Norman, 2004; Langston and Wood, 2009; Chao et al., 2016; Barker and Warburton, 2020) and rule-based rewarded tasks (e.g., Day et al., 2003; Ergorul and Eichenbaum, 2004; Crystal and Smith, 2014). Using spontaneous object exploration tasks different configurations of objects, object position, contexts, and temporal order permit testing of different components of episodic-like memory. As episodic memory formation involves the binding of memory traces for what happened during a specific experience together with the spatial and temporal context in which it occurred, it has been argued that spontaneous object exploration tasks that requiring binding of objects (what), with specific locations (where) and contexts (which occasion) provide a valid model of episodic or episodic-like memory in rodents (Eacott and Norman, 2004; Davis et al., 2013; Ross and Easton, 2021).

Key advantages of spontaneous object exploration tasks, compared to food-rewarded tasks, are that they are based on one-trial learning, and therefore permit testing within acute time windows, and they do not require training that can shape subsequent behaviour of subjects. This is crucial when studying the developmental trajectory of episodic-like memory longitudinally.

Rats have been the rodent model of choice when studying the development of neural circuits that support memory processes (Langston et al., 2010; Wills et al., 2010; Ainge and Langston, 2012; Muessig et al., 2016; Shan et al., 2022). Moreover, genetic rat models are currently making unique contributions in our understanding of the pathophysiology associated with cognitive phenotypes in neurodevelopmental disorders (Till et al., 2015; Asiminas et al., 2019; Marshall et al., 2021). Therefore it is essential to determine the normal developmental trajectory of episodic-like memory in rats, and reconcile this trajectory with the development of neural circuits that are known to support it, in order to provide a basis for comparison with developmental trajectories of episodic-like memory in rat models of neurodevelopmental conditions (Cruz-Sanchez et al., 2020). Given the variety of outbred rat strains currently used, it is also important to test more than one rat strain to account for strain-specific trajectories (Andrews et al., 1995; Clemens et al., 2014; Kumar et al., 2015).

Over the last two decades, several studies have focussed on the ontogeny of various type of object memory in rats (Ainge and Langston, 2012; Westbrook et al., 2014;

Ramsaran et al., 2016a,b; Travaglia et al., 2018; Cruz-Sanchez et al., 2020; Sanders et al., 2020). Overall, these studies agree that the ability of rats to exhibit memory for objects bound to other contextual and/or spatial information emerges later than the memory of objects. However, small methodological differences and/or rat strains makes interpretation of these results challenging.

In the present set of studies, we examined the development of episodic-like object-place-context memory, as well as object memory, object-context memory and object-place memory in three commonly used outbred rat strains: two hooded strains [Long Evans Hooded (LEH) and Lister Hooded (LH)], and one albino strain [Sprague Dawley (SD)]. Together with Wistar rats, these strains represent 95.7% of rat strains used in neuropsychiatric experiments (Noori et al., 2018). Using a longitudinal study design, we first explored the developmental trajectory in these four tasks in LEH and SD rats. Given the different overlapping brain circuits supporting memory in each of these tasks, we predicted that rats would exhibit distinct developmental trajectories across the tasks, but that these trajectories would be similar across strains. To control for the possibility that object memory interference and/or contextual habituation across the course of the longitudinal experiment influences the performance of the rats, we also conducted a cross-sectional study, where different rats were used as subjects at each time point. This was conducted with LH rats, which also allowed us to explore developmental trajectories in a third rat strain.

Materials and methods

Animals

Rats used in all studies were bred in-house and kept on a 12 h light/dark cycle (lights on: 7 a.m.; lights off: 7 p.m.). Adult rat breeding pairs were either purchased from Charles River (LH) or bred in-house (SD, LEH: University of Edinburgh). Litters were culled to eight pups shortly after birth to reduce variance due to unequal maternal attention [except from three litters in the cross-sectional study used in age points P25/26 (10 rats), P31/32 (11 rats), P45/46 (9 rats)]. If the litter was born during the day (between 8 a.m. and 5 p.m.) then that day was taken as postnatal Day 0 (P0), and if the litter was born overnight then the following day was taken as P0. Pups were weaned at P21 and were then kept in same sex groups of 2–5 rats per cage.

For the longitudinal studies with Sprague-Dawley (SD) [n = 16 from seven litters (1–5 rats per litter)] and Long-Evans Hooded (LEH) rats [n = 13 from seven litters (1–3 rats per litter)], the same male rats were used for all testing points. For the cross-sectional study with Lister Hooded (LH) rats [n = 173 from a total of 23 litters (8–11 rats per litter)], male and female rats from a given litter were all assigned to the same testing age group. The choice of testing point was done in a pseudo-random fashion. For details of rats, litters, and testing time points see **Supplementary Table 1**. All animals had unrestricted access to food and water at all times. All animal experiments were approved by the University of Edinburgh or University of Dundee Animal Welfare and Ethical Review Board before their start and were performed in accordance with the guidelines established by European Community Council Directive 2010/63/EU (22 September 2010) and with the Animal Care (Scientific Procedures) Act 1986.

Behavioural tasks

Data collection took place across two labs. The longitudinal datasets from SD and LEH rats were collected at the University of Edinburgh (Wood lab) while the cross-sectional datasets from LH rats were collected at the University of Dundee (Langston lab).

Apparatus and objects

For studies conducted in the Wood lab, animals were tested in a rectangular polycarbonate testing box (76 cm long × 45 cm wide × 60 cm tall) with removable wall and floor inserts that could be rearranged to form two distinct contexts. Context 1 had wooden walls covered with white textured wallpaper and a wood-effect linoleum floor. Context 2 had matt blue painted walls and a black rubber-textured floor. The box remained in the same location within the room for both context configurations. Two 3M Dual-Lock resealable fasteners were attached to the floor, 9 cm from the box walls at north-east and north-west locations, used to keep the two objects firmly attached to the floor in the same locations for every trial. The testing box was situated on a table surrounded on three sides by a black curtain, with one opening at the south side of the box (where subjects were always placed). The distance between the curtains and east and west walls of the testing apparatus was approximately 30 cm. The north wall of the testing apparatus was immediately adjacent to the curtain. Inside the curtained enclosure a lamp situated at the north-east side of the enclosure provided additional light. A multicoloured feather duster just above the north-west corner and a high contrast 3D shopping bag just above the north-east corner provided prominent visual three-dimensional cues; these were hung just above the box but were out of reach of the subjects. These cues remained in the same position and orientation throughout the experiments regardless of which context was being used. The rest of the external environment was also kept as consistent as possible, and a radio on low volume was used to mask potentially distracting noises. An opaque holding bucket (30 cm diameter, 40 cm tall) with bedding inside, which was used to hold rats between trial phases, was placed outside the curtained environment. An overhead black and white camera was used to monitor the

rat in the testing box. The video signal was fed into a DVD recorder and a computer on the desk of the experimenter, which was 2 m away from the testing box. A schematic of the arrangement of the room, curtains and testing box is depicted in **Supplementary Figure 1A**.

For studies conducted in the Langston lab, testing was carried out within a rectangular polycarbonate testing box (58 cm long by 40 cm wide by 47 cm tall) with a wood-effect linoleum floor. The testing box was situated in the corner of the experimental room where it remained throughout all testing procedures. The testing box could be configured to make two different contexts. Context 1 had blue walls with a black perforated rubber mat floor, whereas context 2 had white and black walls with a white plastic grid placed on the linoleum floor. The arena sat on a bench 65 cm above the ground in the corner of the room. A red plastic flower and a large green playing block were used as prominent visual three-dimensional cues and were placed in the north-east and north-west corners of the arena, suspended 40 cm above the arena floor (**Supplementary Figure 2C**). These cues were constantly present irrespective of the contextual configuration of the arena. An opaque holding bucket was placed next to the testing box. The overhead camera was connected to a recording device and computer at the opposite side of the room to the testing box, where the experimenter scored rat object exploration. **Supplementary Figure 1B** shows the arrangement of the testing room, while **Supplementary Figure 1C** provides photographs of the two context configurations and the prominent cues used in the Langston lab.

A variety of objects were used, which were between 8 cm × 8 cm × 8 cm and 11 cm × 11 cm × 11 cm. The objects were non-porous and could be easily cleaned (photographs of all objects are shown in **Supplementary Figure 2**). Each object was paired with another that differed in shape, material, colour, or texture. Analysis of the sample phase explorations pooled across all rats, tasks and time points from the two longitudinal studies confirmed that rats showed similar innate interest to both objects within each pair (**Supplementary Figure 3** and **Supplementary Table 5**). For longitudinal studies, each object-pair was used only once per animal. For the cross-sectional study, the same four object-pairs were used for a given task across all age time points.

Experimental timeline

For the longitudinal study in SD rats, animals were handled in the animal facility for 6 days while still in the cage with their mothers (P16–P21). After weaning they were handled for one day (P22), in the experimental room, such that they received a total of 7 days of handling. Habituation (see below) took place on P23&P24. Behavioural testing (see below) took place on the following pairs of adjacent days: P25&P26, P32&P33, P37&P38, P43&P44, P49&P50, P55&P56, P61&P62, P70&P71.

For the longitudinal study in LEH rats, animals were handled in the animal facility for three days while still in the cage with their mothers (P19–P21). After weaning they were handled for three days in the animal facility (P22–P24) and for one day in the experimental room (P25) to reach a total of 7 days of handling. Habituation took place on P26&P27. Behavioural testing took place on the following pairs of adjacent days: P28&P29, P35&P36, P42&P43, P49&P50, P55&P56, P64&P65.

For the cross-sectional study in LH rats, animals were handled for the 7 days immediately prior to habituation and habituation took place during the 2 days before each testing point. For example, rats tested at the first testing point (P25&P26), were handled and habituated on the same time frame as the SD rats in the longitudinal study, while rats in the second testing point (P31&P32) were handled for 7 days from P22–P28 and habituated on P29&P30. Behavioural testing took place on the following pairs of adjacent days: P25&P26, P31&P32, P33&P34, P34&P35, P38&P39, P42&P43, P45&P46, P47&P48, P50&P51, P70&P71.

Handling and habituation procedures

Handling involved 10 min per day of gently lifting the animals multiple times and allowing them to sit on the experimenters' arms and lap. This allowed rats to get comfortable with the experimenter and the process of being lifted from their home cage. Habituation was performed in the testing box to familiarize the animals to both contextual configurations of the testing box, to the box's location within the stable environment, to the holding bucket that was used during the task. On the morning of the first day of habituation, the animals were placed in each context configuration in cage groups (30 min per context). In the afternoon they were placed individually into each context configuration (10 min per context). Between exposures to context 1 and context 2, rats were placed into the holding bucket for 2 min. On the second day of habituation, animals were individually habituated twice to each context configuration (once to each in the morning and once to each in the afternoon; 10 min per context exposure) but this time, two different objects were fixed in the positions where the rats would encounter objects during testing. These objects were not used again during testing. During the habituation sessions, rats were left undisturbed to explore the contexts and objects. For the cross-sectional study, an identical habituation protocol was used for each group of animals during the two days preceding testing.

Testing procedures

Rats were tested for a single trial on each of four different object exploration tasks over a 2-day testing period (Day l, 8.30 a.m.–12.30 p.m.: object recognition (OR), 2.30 p.m.–6.30 p.m.: object-context recognition (OCR); Day 2, 8.30 a.m.–12.30 p.m.: object-place recognition (OPR), 2.30 p.m.–6.30 p.m.: object-place-context recognition (OPCR). Each trial of each task

consists of multiple "phases": OR and OPR each have one sample phase and one test phase, whereas OCR & OPCR each have two sample phases and one test phase.

Before the start of each trial, copies of the objects needed for that trial were cleaned. For each phase of every task, the experimenter prepared the appropriate context configuration and attached two cleaned objects to the appropriate locations in the box. At the start of each phase, the rat was placed in the testing box from the south side facing the south wall of the apparatus, away from the objects (**Figure 1**). Prior to the first sample phase, the rat was its home cage, between phases, the rat was placed in an opaque holding bucket, and after the test phase it was returned to its home cage. During each phase, the rat was free to explore the objects and the testing box. The sample and test phases were each 3 min long and the interval between phases was 2 min. At the end of each trial and before testing the next rat, the objects and testing environment were cleaned with 70% ethanol solution and unscented baby wipes (Huggies).

Novel object positions, test phase contexts, sample phase context order (in OCR and OPCR), and identity of the object from each object pair that was designated as novel or familiar were counterbalanced across rats, tasks, and (for the longitudinal study only) time points, to ensure that the final results were as unbiased as possible. While all individual parameters were counterbalanced between rats at each time point, not all possible combinations of parameters were counterbalanced within each task at each time point. The counterbalancing overview scheme for the longitudinal testing of SD rats can be found in **Supplementary Table 4**. Similar counterbalancing was used for LEH longitudinal testing. For LH cross-sectional testing the same object pairs were used for every time-point/task.

Object recognition

The OR task consists of two phases: sample and test (**Figure 1A**). In the sample phase, two identical objects are available in either context 1 or context 2. In the test phase, two objects are available in the same context as the sample phase. One object is a duplicate of one of the objects used in the test phase, whereas the other is a novel object. This task is used to test whether the animal can detect object novelty and discriminate between the familiar and novel objects. Higher exploration of the novel than the familiar object is indicative of memory for the familiar object.

Object context recognition

The OCR task consists of three phases: sample 1, sample 2 and test (**Figure 1B**). In sample phase 1, two identical objects are available in either context 1 or context 2. In sample phase 2, a different pair of identical objects is available in the other context. In the test phase, two objects (one is a duplicate of the objects from sample phase 1 and the other is a duplicate of the objects from sample phase 2) are available in either context 1 or context 2. This task is used to test whether an animal can associate an object with a surrounding context. Higher exploration of the object which is in a different context than it was experienced in the sample phase is indicative of OCR memory.

Object place recognition

The OPR task consists of two phases: sample and test (**Figure 1C**). In the sample phase, two non-identical objects are available in either context 1 or context 2. In the test phase, two objects (both duplicates of one of the objects from the sample phase) are available in the same context as in the sample phase. The positions where objects are situated does not change between phases, but the association of object identity and position is. Effectively, this task is used to test whether an animal can associate a specific object with a location in space. Higher exploration of the object that is in a different location than it was experienced in the sample phase is indicative of OPR memory.

Object place context recognition

The OPCR tasks consists of three phases: sample 1, sample 2 and test (**Figure 1D**). In sample phase 1, two non-identical objects are available in either context 1 or context 2. In sample phase 2, duplicates of the same two objects used in sample phase 1 are available, but the objects have swapped locations and are in the other context. In the test phase, two identical objects (further duplicates of one of the two objects from sample phases 1 and 2) are available in one of the two contexts. This task is used to test whether the animal can associate an object with a location in a specific context. Higher exploration of the object which is in a different object-place-context configuration than it was experienced in the sample phase is indicative of OPCR memory.

Scoring and statistical analysis

The time spent exploring each object in each sample phase and each test phase was scored manually using a simple timer computer program, with the experimenter pressing one button for each object to indicate the start and end of exploration. Object exploration was defined as the animal actively exploring an object with its snout within 2 cm of the object and performing actions such as sniffing and whisking. Exploration was not scored when the animal was not actively exploring object (e.g., climbing or resting on an object). To ensure manual scoring uniformity between experimenters and experiments, a subset of data (approximately 200 trials) were re-scored by an experimenter from the other institution (i.e., exploration originally scored "live" in Edinburgh was re-scored from video by SL at Dundee, and exploration originally scored "live" at Dundee was rescored from video by AA at Edinburgh). The re-scoring was conducted with the scorer blind to the age of the rat, to whether they were scoring a sample or a test phase, to the task that the data came from, and to which objects

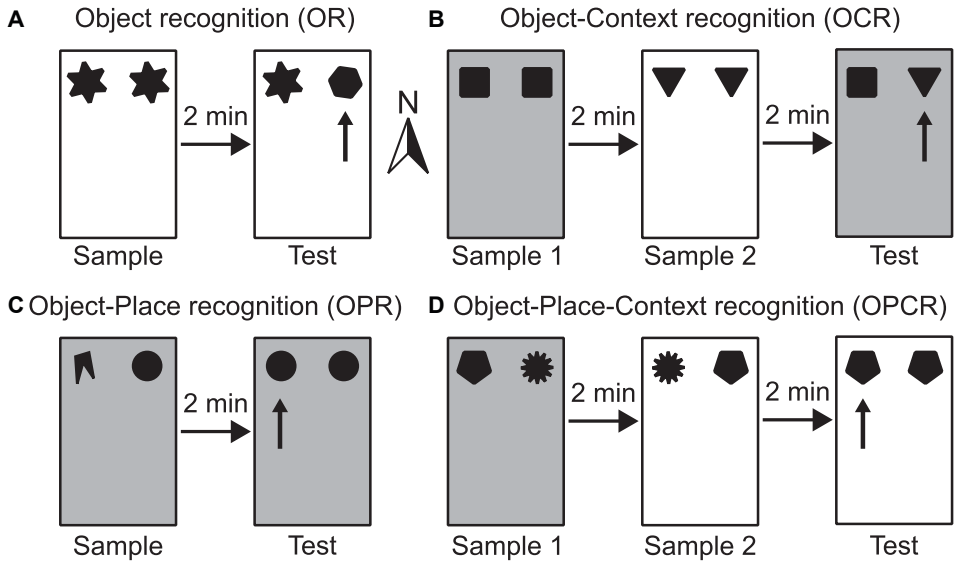

FIGURE 1
Schematic illustrations of example configurations for the four spontaneous object exploration tasks used **(A–D)**. Rectangles depict the testing box, which can be configured either as context 1 (shown as white) or context 2 (shown as grey) by changing floor and wall inserts. The different shapes represent different objects used. **(A)** OR task–the arrow indicates which object is novel in the test phase; **(B)** OCR task–the arrow indicates which objects in a novel configuration with respect to its context in the test phase; **(C)** OPR task–the arrow indicates which object is in a novel configuration with respect to its position in the test phase; **(D)** OPCR task–the arrow indicates which object is in a novel configuration with respect to the combination of position and context in the test phase. Compass arrow indicates conventional north referenced in methods' section.

or object configurations were novel and familiar. Correlation of the discrimination ratios between objects calculated based on the two scorers was highly significant ($R^2 = 0.8321$, $p < 0.001$).

Trials in which animals showed very low object exploration (less than 5 s of exploration of each object in a sample phase or less than 10 s of total object exploration in the test phase) were excluded from analysis. The sample sizes included in the analysis for each testing time point and each study after these exclusion criteria were applied are detailed in the figure legends and collectively shown in **Supplementary Table 1** and **Supplementary Table 2**. The full dataset produced has been included as a supplement to the manuscript (**Supplementary Table 6**). For each test phase, the Discrimination Index (DI) {[(time exploring novel object or object configuration)–(time exploring familiar object or object configuration)]/(time exploring both objects)} was calculated. For all studies, one-sample *t*-tests were used to compare DIs against chance (DI = 0) controlled for the false discovery rate using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

For longitudinal studies, the effects of age and strain on the total sample phase object exploration, total test phase object exploration, and the discrimination index were analyzed for each task by fitting a Linear Mixed Effects (LME) model using Maximum Likelihood (ML). Week of age (week), strain and strain × week interaction were used as fixed factors. Rat identity and litter identity were included as random factors to account for rat and litter specific effects (Bates et al., 2015; Golub and Sobin, 2020; Yu et al., 2022). For instances where the LME-ML indicated a significant strain × week interaction, two-sample *t*-tests were conducted to compare the two strains at each time point (controlled for the false discovery rate using the Benjamini-Hochberg procedure). For these analyses, data for each rat strain were binned into different approximate "weeks of age" of the rats, to circumvent the small inconsistencies in testing days between strains. Specifically, the data points between P28–P33 were defined as 4 weeks old, P35–P38 as 5 weeks old, P42–P44 as 6 weeks old, P49&P50 as 7 weeks old, P55–P57 as 8 weeks old, and P61–P65 as 9 weeks old. As only the SD rats were tested younger than 4 weeks old (P25&P26) and older than 9 weeks old (P70&P71) these data points were not included in the LME-ML analyses. For the cross-sectional study, a LME-ML model was used to examine the effect of age on the discrimination index, total sample phase exploration, and test phase object exploration, for each task. Age was set as the only fixed factor, with rat identity as random factor, and sex-within-litter identity as a nested random factor. A two-way ANOVA was used to explore the effects of sex. Rats were used as the experimental unit in all main analyses presented in this manuscript. However, we also analyzed data using a more stringent approach aiming at eliminating intra-litter statistical correlations. For the longitudinal study on SD and LEH rats, we also analyzed the data using litter as the experimental unit. One-sample *t*-tests were used to compare litter-averaged

discrimination index data against chance (DI = 0). For the cross-sectional study, data were averaged across rats of the same sex and litter. One-sample *t*-tests were used to compare litter-averaged discrimination index data for each sex against chance (DI = 0). Statistical analyses were performed using GraphPad (Prism 9.3), MATLAB (version R2021b; Mathworks), and R 4.1.2 (RStudio Team, 2021). Probabilities of $p < 0.05$ were considered as significant. Data are presented as means with error bars denoting the standard error. Distributions from all studies, tasks and ages were tested for normality using Kolmogorov-Smirnov test. Almost all distributions passed the normality test (Exceptions: Longitudinal: OCR: SD-P33, OPR: SD-P62, LEH-P57, OPCR: SD-P33, LEH-P43; Cross-sectional OR: LH-P70, OPR: LH-P48, OPCR LH-P35, LH-P48). For these time points the Wilcoxon Signed Rank test was used to test difference from chance performance (DI = 0).

Results

Object recognition is evident at all ages tested

Both LEH and SD rats exhibited strong preference for novel over familiar objects in the OR task at every time point, indicated by discrimination indices that were significantly higher than the chance level of zero (**Figure 2B** and **Supplementary Figures 4A, 5A** for individual animal data; one-sample *t*-tests vs. chance $p < 0.05$ for all time points in both strains). This indicates intact object recognition memory from the earliest age tested (P25 in SD, P28 in LEH) and at all subsequent ages. To compare the DIs directly between strains and across ages, the data were binned into 6 "week of age" bins (from ∼4 weeks to ∼9 weeks old), because the exact postnatal day of testing differed between the two strains (see section "Materials and methods" for details of bins). The different week of age bins are indicated on **Figures 2B–D** with vertical shading. A LME-ML analysis was used to compare the two strains (LEH and SD) across the different week of age bins. This revealed no significant differences between the two strains ($F_{(1,\ 27)} = 1.948$, $p = 0.174$) or between weeks ($F_{(5,\ 132)} = 0.897$, $p = 0.485$ and the strain × week interaction was also not significant ($F_{(5,\ 132)} = 0.912$, $p = 0.476$). Together, these analyses indicate stable and significant object recognition memory in SD and LEH rats from 3–4 weeks old.

We next tested whether there were differences in the total amount of object exploration in the sample and test phases across strains and week of age, as the amount of sample phase exploration can impact object recognition memory. Analysis of total sample phase object exploration revealed no significant main effects of strain (**Figure 2C**; LME-ML: $F_{(1,\ 27)} = 0.763$, $p = 0.390$) or week of age ($F_{(5,\ 132)} = 1.841$, $p = 0.083$), but there was a significant strain × week interaction ($F_{(5,\ 132)} = 2.448$, $p = 0.037$). However, *post-hoc* testing (multiple two sample *t*-tests with correction for multiple comparisons) did not reveal significant differences between the two strains at any age. Total test phase object exploration also did not differ between strains (**Figure 2D**; LME-ML: $F_{(1,\ 27)} = 0.011$, $p = 0.916$). However, we found a significant main effect of week ($F_{(5,\ 132)} = 4.401$, $p = 0.001$) with no significant strain × week interaction ($F_{(5,\ 133)} = 1.134$, $p = 0.346$).

Finally, to test whether there was an association between total sample or test phase exploration and object recognition memory, we analyzed the correlations between these variables and the discrimination index (collapsing across the different ages). This revealed a significant negative correlation between DI and both sample and test phase object exploration in SD rats (DI vs. Sample: $R = -0.279$, $p < 0.01$; DI vs. Test: $R = -0.444$, $p < 0.001$) and no significant correlations for LEH rats (**Supplementary Table 3**). A negative correlation between sample phase exploration and DI is surprising, as previous studies have shown that greater sample phase exploration generally leads to enhanced memory (Cohen and Stackman, 2015). Given the relatively high levels of sample phase exploration and good object recognition performance at all ages (and in both strains), we think it is unlikely that variance in sample phase exploration is influencing memory performance in the current experiment. In contrast, a negative correlation between total test phase exploration and DI might be predicted if animals explore well remembered familiar objects less than poorly remembered familiar objects. If this is the case, good memory (reflected as a higher DI) would result in lower total test phase exploration. As this relationship was only observed in the SD rats, yet object recognition memory was similar across strains, we would conclude that variability in test phase exploration is unlikely to be influencing memory performance. Together, our findings on the OR task are consistent with our previous findings as well as work from other laboratories suggesting that the ability to recognize objects emerges before the third week of life in rats (Reger et al., 2009; Ainge and Langston, 2012; Westbrook et al., 2014; Cruz-Sanchez et al., 2020).

Object-context memory emerges around 5 weeks of age

The ability to discriminate novel from familiar object-context associations was first seen at around 5 weeks of age in both LEH and SD rats, indicated by discrimination indices that were significantly higher than the chance for all time points from five weeks old, but not at earlier time points (**Figure 3B** and **Supplementary Figures 4B, 5B** for individual animal data; one-sample *t*-tests vs. chance levels $p < 0.05$ for all time points from P35 in LEH and P37 in SD rats). Further analyses revealed

a significant main effect of strain (LME-ML; $F_{(1,\ 27)} = 4.57$, $p = 0.041$) and a significant main effect of week ($F_{(5,\ 134)} = 3.31$, $p = 0.008$) but no significant strain × week interaction ($F_{(5,\ 134)} = 0.702$, $p = 0.623$). The significant difference in DI between strains reflects consistently higher discrimination ratios in LEH than in SD rats. However, given that discrimination is better than chance for both strains from week five onwards, there is no indication that the time course of development of OCR memory differs between genotypes. Rather, these analyses indicate that significant object-context recognition memory emerges sometime between 4 and 5 weeks old in both SD and LEH rats, after which it is expressed consistently.

Analysis of the total sample phase object exploration revealed no significant main effect of strain (Figure 3C; LME-ML: $F_{(1,\ 27)} = 1.455$, $p = 0.238$), but a significant main effect of week ($F_{(5,\ 134)} = 10.528$, $p < 0.001$) and a significant strain × week interaction ($F_{(5,\ 134)} = 7.561$, $p < 0.001$). *Post-hoc* tests indicated that the strains differed significantly only on week seven, where LEH rats had higher sample phase object exploration than SD rats. Analysis of the total test phase object exploration revealed no significant main effect of strain (LME-ML: $F_{(1,\ 27)} = 3.33$, $p = 0.079$), but again there was a significant main effect of week ($F_{(5,\ 134)} = 4.419$, $p < 0.001$) while the week × strain interaction was not significant (week × strain $F_{(5,\ 134)} = 2.077$, $p = 0.072$) (Figure 3D). Although both sample and test phase object exploration showed a significant main effect of week, there was no consistent trend in total object exploration over weeks for either strain, unlike the more consistent trajectory of the discrimination ratio in both strains. Correlation analyses between DI and both sample and test

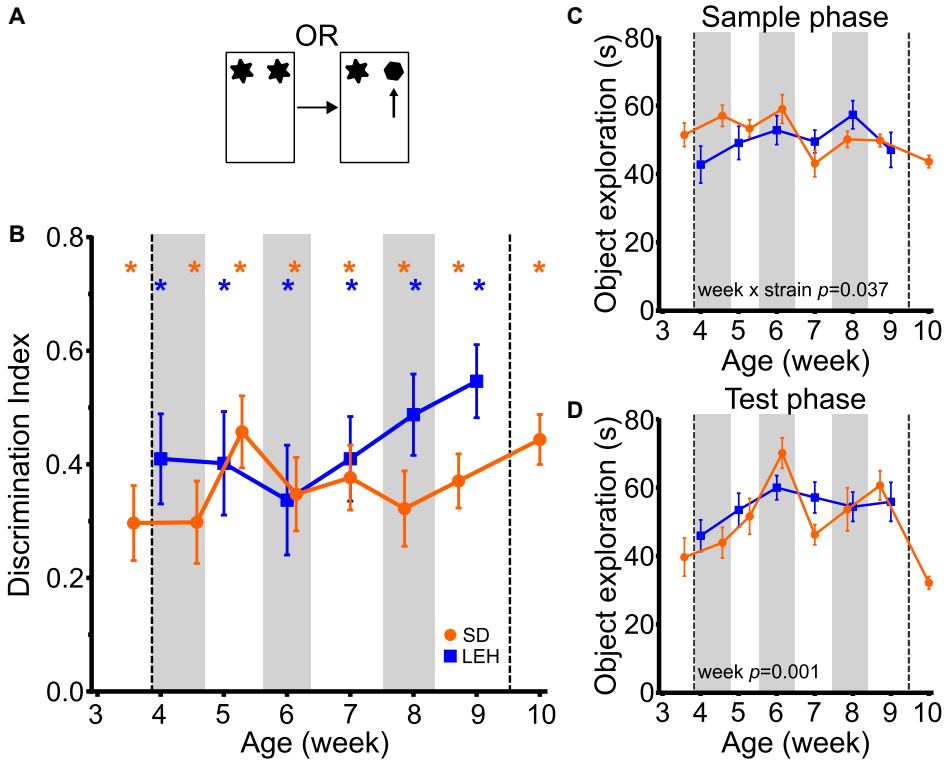

FIGURE 2
Object recognition memory is evident from the fourth week of age in LEH and SD rats. **(A)** Schematic of object recognition task; **(B)** Object recognition memory performance, expressed as a discrimination index across development. Both LEH and SD rats show significant memory from the first time tested (3rd week of age (P25) for SD and 4th week of age (P28) for LEH)–blue and orange asterisks indicate significant difference from chance (DI = 0) for LEH and SD rats respectively, based on one-sample *t*-tests. *$p < 0.05$. To compare directly between strains and across ages, the data were binned into 6 "week of age" bins (from ∼4 weeks to ∼9 weeks old), because the exact postnatal day of testing differed between the two strains (see section "Materials and methods" for details of bins). The different week of age bins are indicated on panels **(B–D)** with alternating vertical shading, with the first grey shaded area corresponding to 4 weeks old. As only the SD rats were tested younger than 4 weeks old (P25&P26) and older than 9 weeks old (P70&P71) these data points were not included in the cross-species comparisons (vertical dashed lines). The *p*-values for significant main effects or interactions from the LME-ML analyses between strains and weeks of age are stated within each graph. For OR memory, no significant effects of strain, week or strain × week interactions were detected; **(C)** Object exploration during sample phase for each testing time point for both LEH and SD rats. A significant week × strain interaction was detected; **(D)** Object exploration during test phase for each testing time point for both LEH and SD rats. Only a significant effect of week was detected. *p*-values from one-sample *t*-tests have been corrected for false discovery rate using the Benjamini-Hochberg procedure. [SD]: $n = 16$ for all time points; [LEH]: $n = 13$ except P35&P64 where $n = 12$. For details on sample sizes, *t*, and *p*-values for one-sample *t*-tests, see **Supplementary Table 2**.

phase total object exploration times revealed no significant correlations for SD, and a positive correlation between DI and sample phase (but not test phase) object exploration for LEH [Supplementary Table 3; (LEH) DI vs. Sample: $R = 0.36$, $p < 0.001$]. As discussed above, more sample phase exploration could result in better memory. However, as this relationship was seen only in LEH rats, and the developmental emergence of OC recognition did not differ between strains, we think variability in sample phase exploration is unlikely to account for time course of the emergence of object-context memory. Overall, these results indicate that the ability to recognise object-context associations develops around 5 weeks of age, later than the ability to recognise objects.

Object-place memory emerges around seven weeks of age

Both SD and LEH rats can discriminate between novel and familiar object-place associations only from around 7 weeks of age, indicated by discrimination indices that were significantly higher than chance for all time points from seven weeks old, but not at earlier time points (Figure 4B and Supplementary Figures 4C, 5C for individual animal data; one-sample t-tests vs. chance levels $p < 0.05$ for all time points from P50 in both LEH and SD rats). Further analysis revealed no significant difference between the strains (LME-ML: $F_{(1, 27)} = 1.468$, $p = 0.236$) but a significant main effect of week ($F_{(5, 134)} = 12.44$, $p < 0.0001$), and no significant interaction between strain and week ($F_{(5, 134)} = 1.957$, $p = 0.089$). These data suggest that OPR memory emerges around seven weeks of age in both SD and LEH rats.

Analysis of total sample phase object exploration indicated no significant difference between the strains (Figure 4C; LME-ML: $F_{(1, 27)} = 0.030$, $p = 0.863$) but there was a significant main effect of week ($F_{(5, 134)} = 3.193$, $p = 0.009$), and a significant strain × week interaction ($F_{(5, 134)} = 4.84$, $p < 0.001$). However, *post-hoc* tests indicated that the strains did not differ significantly from one another at any time point. There was no main effect of strain (LME-ML: $F_{(1, 27)} = 1.139$, $p = 0.295$) or week ($F_{(5, 134)} = 0.443$,

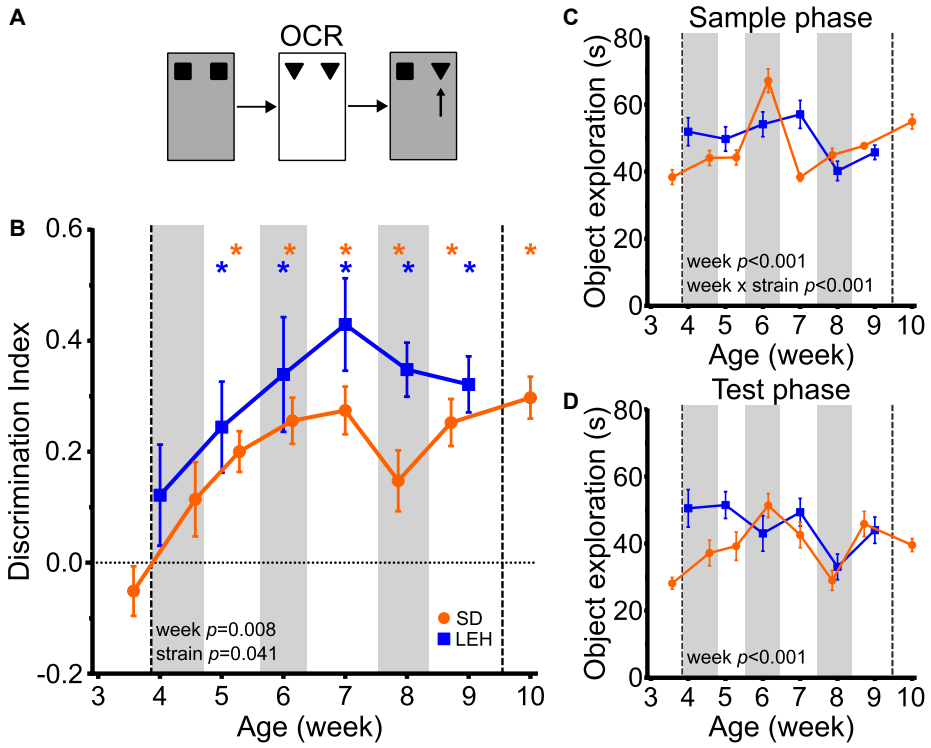

FIGURE 3
Object-context recognition memory emerges at around 5 weeks of age in LEH and SD rats. **(A)** Schematic of object-context recognition task; **(B)** Object-context recognition memory performance, expressed as a discrimination index across development. Both LEH and SD rats show significant memory at all time points from the 5th week of age (P35 for LEH, P37 for SD). *$p < 0.05$. Significant main effect of week and strain were found; **(C)** Object exploration during the sample phases (mean in the two sample phases) for both LEH and SD rats. A significant main effect of week and a significant week × strain interaction were detected; **(D)** Object exploration during the test phase. A significant main effect of week was detected. [SD]: $n = 16$ for all time points; [LEH]: $n = 13$ except P49 where $n = 12$. For details on sample sizes, t, and p values for one-sample t-tests, see Supplementary Table 2. Asterisks, shading on graphs etc., follow same convention as Figure 2.

p = 0.818) for total test phase object exploration, but a significant strain × week interaction was found (Figure 4D; $F_{(5,\ 134)}$ = 2.444, p = 0.037). *Post-hoc* tests indicated that the strains differed significantly in total test phase object exploration only on week nine, where SD animals explored objects significantly more than LEH. Correlation analyses for OPR revealed no significant correlations between DI and object exploration during either sample or test phase for SD or LEH rats (Supplementary Table 3). Therefore, there is no indication that the fluctuations in sample phase or test phase object exploration, which were both consistently high, can account for the emergence of significant OPR memory at 7 weeks.

Object-place-context memory emerges around seven weeks of age

Object-place-context recognition memory showed a very similar developmental time course as OPR memory. Discrimination ratios were significantly higher than chance (one-sample t-tests vs. chance levels $p < 0.05$) for all time points from P50 in LEH and SD rats except for P56 in SD rats (Figure 5B and Supplementary Figures 4D, 5D for individual animal data). Further analyses indicated that there was no significant difference between the strains (LME-ML: $F_{(1,\ 27)}$ = 0.454, p = 0.506), but there was a significant main effect of week ($F_{(5,\ 131)}$ = 6.7, $p < 0.0001$) with no significant strain × week interaction ($F_{(5,\ 131)}$ = 0.921, p = 0.469). Thus, like OPR memory, OPCR memory emerges at around 7 weeks of age in both SD and LEH rats.

Analysis of total sample phase object exploration revealed no significant main effect of strain (Figure 5C; $F_{(1,\ 27)}$ = 2.071, p = 0.162), but a significant main effect of week ($F_{(5,\ 131)}$ = 9.906, $p < 0.0001$) and a significant strain × week interaction ($F_{(5,\ 131)}$ = 3.128, p = 0.011). However, *post-hoc* testing indicated that there were no significant differences between strains at any time point, and both strains exhibited high levels of sample phase object exploration throughout testing. Total test phase object exploration also remained high throughout the experiment with no significant main effects of strain

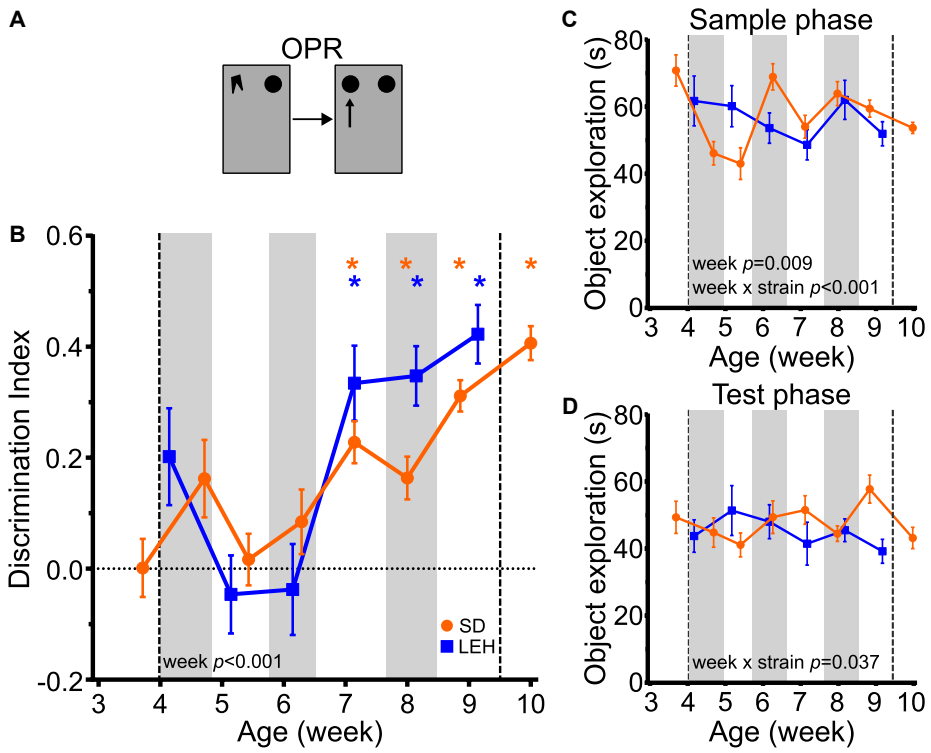

FIGURE 4
Object-place recognition memory emerges at around 7 weeks of age in LEH and SD rats. **(A)** Schematic of object-place recognition task; **(B)** Object-place recognition memory performance, expressed as discrimination index across development. Both LEH and SD rats show significant memory consistently from the 7th week of age (P50 for both LEH and SD). *$p < 0.05$. A significant main effect of week was found; **(C)** Object exploration during the sample and test phases for each testing time point for both LEH and SD rats. A significant main effect of week as well as a significant week × strain interaction were detected; **(D)** Object exploration during the test phase. A significant week × strain interaction was found. [SD]: n = 16 for all time points; [LEH]: n = 13 except P50 where n = 12. For details on sample sizes, t, and p values for one-sample t-tests, see Supplementary Table 2. Asterisks, shading on graphs etc., follow same convention as Figure 2.

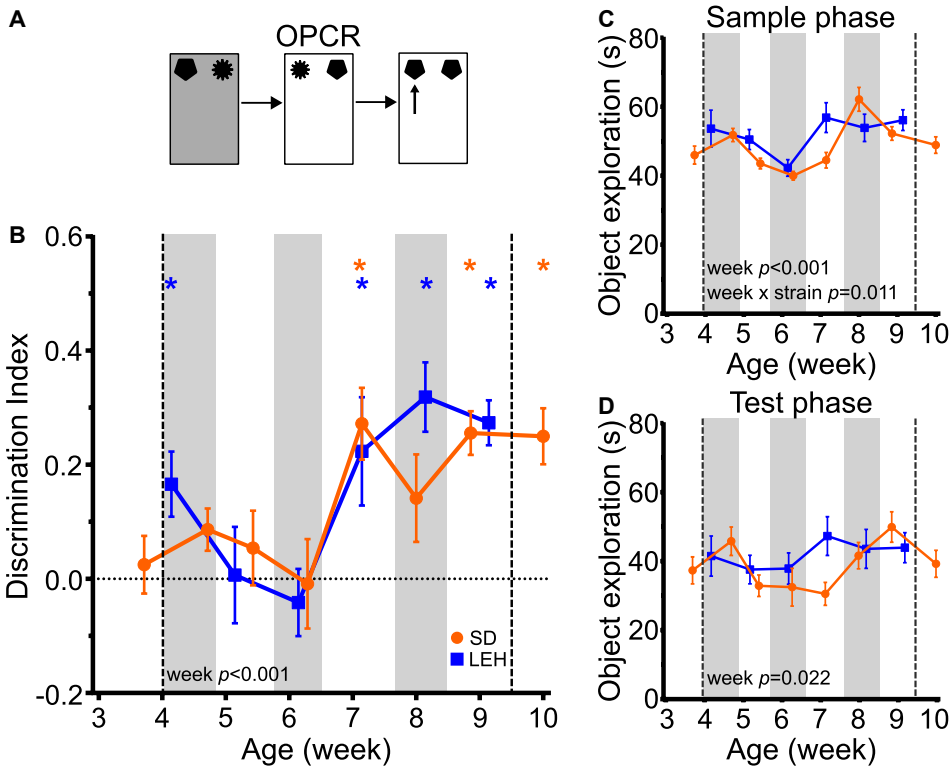

FIGURE 5
Object-place-context recognition memory emerges at around 7 weeks of age in LEH and SD rats. **(A)** Schematic of object-place-context recognition task; **(B)** Object-place-context recognition memory performance, expressed as discrimination index across development. Both LEH and SD rats show significant memory consistently from the 7th week of age (P50 for both LEH and SD), although LEH rats also show significant memory at 4 weeks of age (P39) before performing at chance on weeks five and six. *$p < 0.05$. A significant main effect of week was found; **(C)** Object exploration during the sample phases (mean of the two sample phases) for each testing time point for both LEH and SD rats. A significant main effect of week and a significant week × strain interaction were detected for mean object exploration during the sample phases; **(D)** Object exploration during the test phase. No significant main effects or interactions were detected. [SD]: $n = 16$ except P38&P50 where $n = 15$; [LEH]: $n = 13$ except P43&P57 where $n = 12$. For details on sample sizes, t, and p-values for one-sample t-tests, see **Supplementary Table 2**. Asterisks, shading on graphs etc., follow same convention as **Figure 2**.

($F_{(1,\ 27)} = 0.831$, $p = 0.37$), or week ($F_{(5,\ 131)} = 2.73$, $p = 0.022$), and no significant strain × week interaction (**Figure 5D**; $F_{(5,\ 131)} = 1.75$, $p = 0.129$). Correlation analyses revealed only a significant negative correlation between DI and test phase object exploration for SD rats (**Supplementary Table 3**; DI vs. Test: $R = -0.286$, $p < 0.01$), and no significant correlations between DI and object exploration for LEH. As discussed earlier, we have no reason to expect that a decrease in test phase object exploration would promote OPCR memory. Moreover, the similar developmental trajectory of OPCR memory in SD and LEH rats, and the absence of significant strain × week interaction in test phase object exploration suggest that the variation in test phase object exploration in SD rats is unlikely to be contributing to the developmental emergence of OPCR memory at 7 weeks. Taken together, these data indicate that OPCR memory emerges around the 7th week of age in both strains, and this is unlikely to be secondary to the fluctuations in object exploration in sample and test phases.

Overall, longitudinal testing revealed distinct developmental trajectories across the four spontaneous object exploration tasks in an albino and hooded strain (LME-ML, SD: task × age $F_{(15,\ 298)} = 1.756$, $p = 0.040$; LEH: $F_{(15,\ 234)} = 1.848$, $p = 0.029$). Object recognition memory emerged before four weeks of age, object-context memory emerged at around 5 weeks, while object-place and object-place-context memories emerged around 7 weeks of age.

Despite the spontaneous nature of object exploration tasks, it is plausible that repeated testing over juvenile development leads to context and object memory interference, as well as gradual context habituation, both of which could influence the developmental trajectory of memory in the different tasks. To address this possibility, we conducted a separate cross-sectional study, in which rats were tested at only one time point (age) on the four tasks. For this study we used both males and females from a different hooded rat strain (Lister Hooded–LH). LH rats have been shown to have very similar performance in visual and spatial tasks to LEH rats (Kumar et al., 2015).

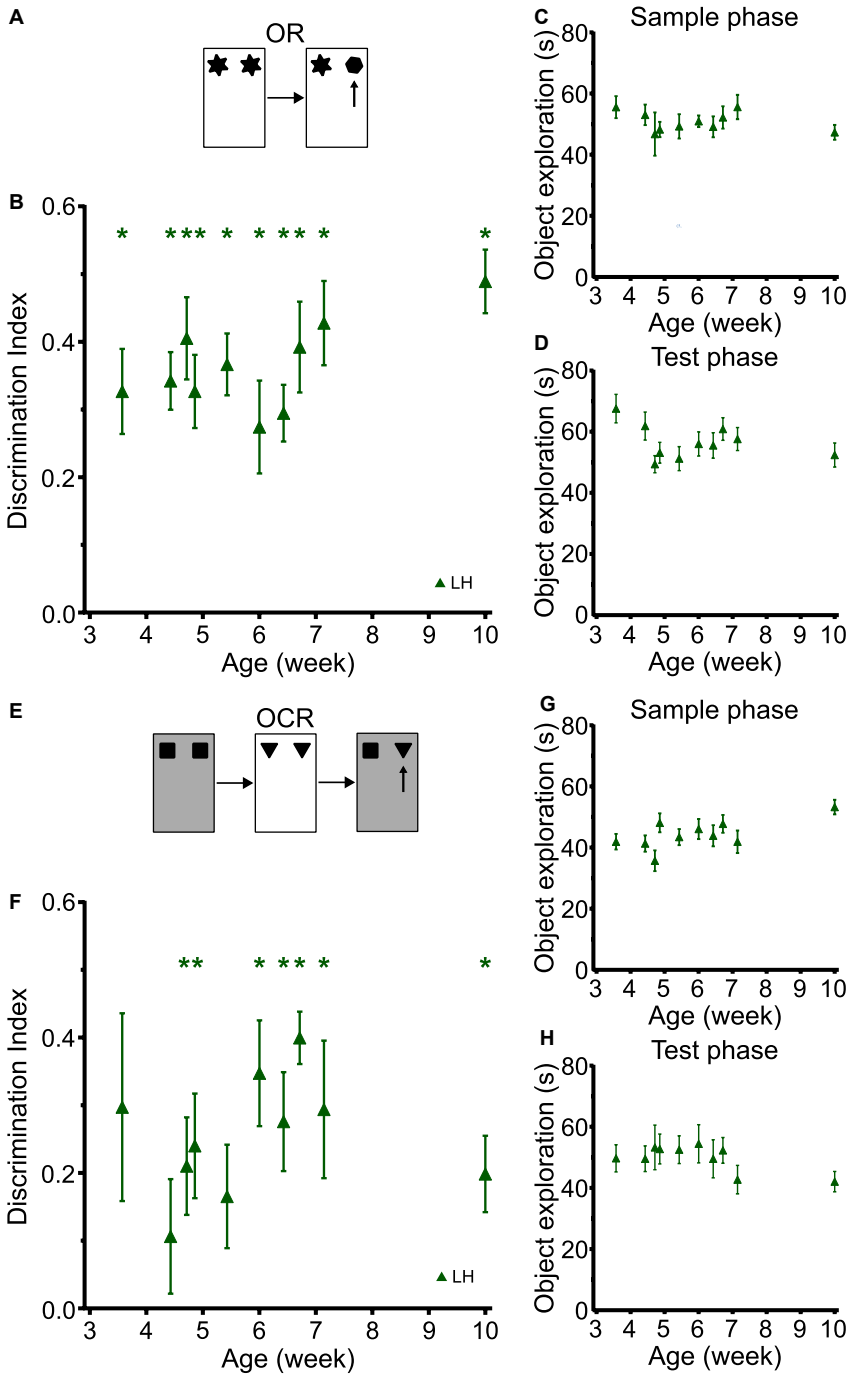

FIGURE 6
LH rats tested in a cross-sectional study design exhibit similar developmental trajectories for object and object-context memory to LEH and SD rats. **(A)** Schematic of object recognition task; **(B)** Object recognition memory performance, expressed as discrimination index, for different cohorts of rats tested at different ages. Green asterisks indicate significant difference from chance (DI = 0), based on one-sample *t*-tests. LH rats show significant object recognition memory from the first time tested (P25) *$p < 0.05$. No significant effect of age was detected; **(C)** Object exploration during sample phase. No significant effect of age was detected; **(D)** Object exploration during test phase. No significant effect of age was detected; **(E)** Schematic of object-context recognition task; **(F)** Discrimination indices for object-context recognition task. LH rats show significant object-context recognition memory from P33 onwards, except at P42; **(G)** Object exploration during the sample phases of the OCR task (mean of the two sample phases). A significant main effect of age was detected; **(H)** Object exploration during test phase of the OCR task. There were no significant differences across age. [OR]: P25 ($n_{(females)}$ = 8, $n_{(males)}$ = 10), P31 ($n_{(females)}$ = 13, $n_{(males)}$ = 14), P33 ($n_{(females)}$ = 4, $n_{(males)}$ = 4), P34 ($n_{(females)}$ = 6, $n_{(males)}$ = 10), P38 ($n_{(females)}$ = 6, $n_{(males)}$ = 9), P42 ($n_{(females)}$ = 8, $n_{(males)}$ = 8), P45 ($n_{(females)}$ = 8, $n_{(males)}$ = 9), P47 ($n_{(females)}$ = 7, $n_{(males)}$ = 9), P50 ($n_{(females)}$ = 7, $n_{(males)}$ = 8), P70 ($n_{(females)}$ = 8, $n_{(males)}$ = 8); [OCR]: P25 ($n_{(females)}$ = 8, $n_{(males)}$ = 8), P31 ($n_{(females)}$ = 11, $n_{(males)}$ = 13), P33 ($n_{(females)}$ = 4, $n_{(males)}$ = 4), P34 ($n_{(females)}$ = 6, $n_{(males)}$ = 9), P38 ($n_{(females)}$ = 5, $n_{(males)}$ = 9), P42 ($n_{(females)}$ = 8, $n_{(males)}$ = 8), P45 ($n_{(females)}$ = 8, $n_{(males)}$ = 9), P47 ($n_{(females)}$ = 7, $n_{(males)}$ = 7), P50 ($n_{(females)}$ = 7, $n_{(males)}$ = 8), P70 ($n_{(females)}$ = 8, $n_{(males)}$ = 8). For details on sample sizes, *t*, and *p*-values for one-sample *t*-tests, see **Supplementary Table 2**.

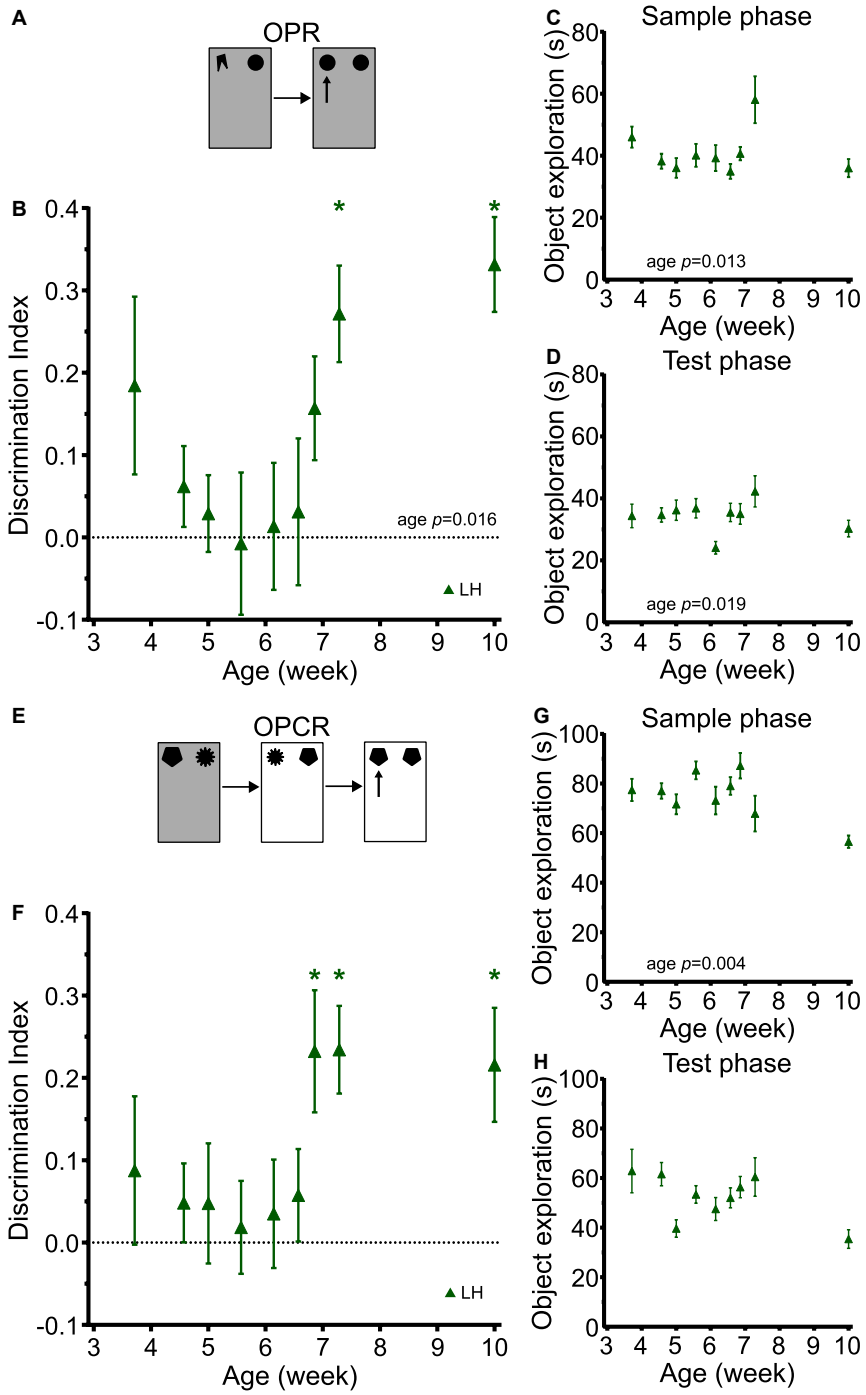

FIGURE 7
LH rats exhibit similar developmental trajectories for object-place and object-place-context memory to LEH and SD rats. **(A)** Schematic of object-place recognition task; **(B)** Object-place recognition memory performance, expressed as discrimination index for different cohorts of rats tested at different ages. LH rats show significant object place recognition memory only on P51 and P71. *$p < 0.05$. A significant main effect of age was found; **(C)** Object exploration during OPR sample phase. A significant main effect of age was detected; **(D)** Object exploration during test phase. A significant main effect of age was detected; **(E)** Schematic of object-place-context recognition task. **(F)** Discrimination indices for Object-place-context recognition memory performance. LH rats show significant object place context recognition memory only on P48, P51, and P71. No significant effect of age was detected; **(G)** Object exploration during OPCR sample phases (mean of the two sample phases). A significant main effect of age was detected; **(H)** Object exploration during OPCR test phase. A significant main effect of age was detected. [OPR]: P26 ($n_{(females)}$ = 8, $n_{(males)}$ = 9), P32 ($n_{(females)}$ = 13, $n_{(males)}$ = 14), P35 ($n_{(females)}$ = 6, $n_{(males)}$ = 9), P39 ($n_{(females)}$ = 6, $n_{(males)}$ = 10), P43 ($n_{(females)}$ = 8, $n_{(males)}$ = 8), P46 ($n_{(females)}$ = 8, $n_{(males)}$ = 9), P48 ($n_{(females)}$ = 7, $n_{(males)}$ = 9), P51 ($n_{(females)}$ = 7, $n_{(males)}$ = 8), P71 ($n_{(females)}$ = 8, $n_{(males)}$ = 8); [OPCR]: P26 ($n_{(females)}$ = 8, $n_{(males)}$ = 8), P32 ($n_{(females)}$ = 13, $n_{(males)}$ = 14), P35 ($n_{(females)}$ = 6, $n_{(males)}$ = 10), P39 ($n_{(females)}$ = 6, $n_{(males)}$ = 10), P43 ($n_{(females)}$ = 6, $n_{(males)}$ = 6), P46 ($n_{(females)}$ = 8, $n_{(males)}$ = 8), P48 ($n_{(females)}$ = 7, $n_{(males)}$ = 8), P51 ($n_{(females)}$ = 6, $n_{(males)}$ = 6), P71 ($n_{(females)}$ = 7, $n_{(males)}$ = 8). For details on sample sizes, *t*, and *p*-values for one-sample *t*-tests, see **Supplementary Table 2**.

Cross sectional testing in Lister Hooded rats reveals similar developmental trajectories as longitudinal testing in Long Evans Hooded and Sprague Dawley rats

In the cross-sectional study with LH rats, object recognition memory was observed at the earliest time point tested (P25) which is similar to what we observed in the longitudinal studies in LEH and SD rats (**Figure 6B** and **Supplementary Figure 6** shows individual animal data for all four tasks; strain: $F_{(2,\ 59)} = 0.655$, $p = 0.523$; strain × age: $F_{(8,\ 196)} = 0.779$, $p = 0.622$). LH rats had discrimination indices significantly better than chance at all time-points (one-sample *t*-tests vs. chance levels $p < 0.05$ for all time points), and there was no significant main effect of age (LME-ML: $F_{(9,\ 153)} = 1.31$, $p = 0.238$). Moreover, rats of all ages showed consistently high sample and test phase object exploration with no significant differences between rats tested at different ages either in sample phase object exploration (**Figure 6C**; LME-ML: $F_{(9,\ 120)} = 0.678$, $p = 0.727$) or in test phase object exploration (**Figure 6D**, $F_{(9,\ 120)} = 1.656$, $p = 0.107$).

The developmental trajectory of OCR memory for LH rats tested cross-sectionally was also similar to that found in SD and LEH rats tested longitudinally (**Figure 6F**; strain: $F_{(2,\ 59)} = 1.098$, $p = 0.340$; strain × age: $F_{(8,\ 194)} = 0.320$, $p = 0.958$). Rats tested at P25 and P31 did not show significant memory ($p > 0.05$), but from P33 rats exhibited above chance discrimination at all ages except for P38 (one-sample *t*-tests vs. chance levels $p < 0.05$ for all time points from P33 except P38). We did not find any significant main effect of age in the OCR discrimination ratios (LME-ML: $F_{(9,\ 111)} = 1.12$, $p = 0.356$). Further analysis revealed no significant main effect of age in either the total sample phase object exploration (**Figure 6G**; LME-ML: $F_{(9,\ 111)} = 1.96$, $p = 0.051$), or the total test phase object exploration (**Figure 6H**; LME-ML: $F_{(9,\ 144)} = 0.687$, $p = 0.719$). The similar trajectories of OC memory in the longitudinal and cross-sectional studies suggest that exposure to multiple objects and repeated exposure to the contexts had negligible effect on developmental trajectories observed in the longitudinal studies.

Object-place recognition memory showed a clear developmental trajectory (**Figure 7B**). Despite the differences in study design and rat strain, this trajectory was very similar to those in SD and LEH rats in the longitudinal study (strain: $F_{(2,\ 252)} = 1.284$, $p = 0.279$; strain × age: $F_{(8,\ 252)} = 1.009$, $p = 0.429$), with LH rats exhibiting discrimination indices significantly above chance levels from P51 (just over 7 weeks old) but not at earlier time points (**Figure 7B**; one-sample *t*-tests vs. chance levels $p < 0.05$ for all ages from P51). A significant main effect of age was found for OPR memory (LME-ML: $F_{(8,\ 115)} = 2.48$, $p = 0.016$). The sample phase and test phase object exploration was high throughout the experiment (>20 s all time points) (**Figures 7C,D**). However, our analysis revealed a significant main effect of age on object exploration during both the sample phase (LME-ML: $F_{(8,\ 115)} = 2.567$, $p = 0.013$) and the test phase (LME-ML: $F_{(8,\ 115)} = 2.42$, $p = 0.019$).

Correlation analyses revealed a significant positive correlation between DI and sample phase object exploration (**Supplementary Table 3**; DI vs. Sample: $R = 0.173$, $p = 0.031$). This raises the possibility that increased sample phase exploration in LH rats may contribute, at least in part, to the OPR performance at some ages. From inspection of the sample phase object exploration data, it appears that exploration in the rats tested at P51 was higher than that of rats tested at earlier time points, and this is the first day on which significant OP memory was observed. However, we think it unlikely that this can account for the emergence of OP memory for two reasons. First, the group tested on P71 showed comparable sample phase object exploration as those tested at earlier time points (i.e., before OP memory was observed), yet still showed significant OP memory. Second, there was no significant correlation between sample phase object exploration and OP memory in the longitudinal studies with SD and LEH rats, where the time course for the emergence of OPR memory was identical. Therefore, it is more parsimonious to conclude that OP memory emerges at around the same point in the three strains of rats independent of sample phase object exploration fluctuations, rather than proposing that in the LH rats it is due to increased object exploration, but that in the LEH and SD rats it is due to some other variable.

The developmental trajectory of OPCR memory in the LH rats tested cross-sectionally was also very similar to those in SD and LEH rats in the longitudinal study (strain: $F_{(2,\ 244)} = 0.104$, $p = 0.902$; strain × age: $F_{(8,\ 244)} = 10.517$, $p = 0.843$), with LH discrimination indices significantly above chance levels from P48 (7 weeks old) but not at earlier time points (**Figure 7F**; one-sample *t*-tests vs. chance levels $p < 0.05$ for all ages from P48 in OPCR). No significant main effect of age was detected for OPC discrimination performance (LME-ML: $F_{(8,\ 105)} = 1.77$, $p = 0.092$). The total object exploration during testing was high for all rat ages. However, our analysis revealed a significant main effect of age on object exploration during both the sample phase (**Figure 7G**; LME-ML: $F_{(8,\ 105)} = 3.08$, $p = 0.004$) and test phase (**Figure 7H**; LME-ML: $F_{(8,\ 105)} = 1.826$, $p = 0.080$). Correlation analyses revealed no significant correlations between DI and sample or test phase exploration. Overall, these analyses suggest that the emergence of memory for object-place-context associations at around 7 weeks of age is not due to changes in object exploration during the sample or test phases.

For the cross-sectional study we used both female and male LH rats. Importantly, our analyses revealed no significant

differences between sexes or significant age × sex interactions for DIs any of the four tasks [2-way ANOVA (OR): Sex: $F_{(1, 143)} = 1.274$, $p = 0.277$; Sex-Age interaction: $F_{(9, 143)} = 0.893$, $p = 0.534$; (OCR): Sex: $F_{(1, 134)} = 1.790$, $p = 0.183$; Sex-Age interaction: $F_{(9, 134)} = 0.427$, $p = 0.919$; (OPR): Sex: $F_{(1, 137)} = 2.738$, $p = 0.10$; Sex-Age interaction: $F_{(8, 137)} = 1.928$, $p = 0.061$; (OPCR): Sex: $F_{(1, 127)} = 0.109$, $p = 0.742$; Sex-Age interaction: $F_{(8, 127)} = 1.195$, $p = 0.307$].

Finally, in addition to our main statistical analyses in which litter identity was fitted as random factor, we performed a more stringent set of analyses in which discrimination indices were averaged across all rats from the same litter (SD & LEH) in the longitudinal study, or across all rats of the same sex from the same litter in the cross sectional study (LH) (**Supplementary Figures 7–9**). This was to ensure maximum control for intra-litter correlations. While these analyses were underpowered, the results were largely consistent with those of our main analyses. Litters performed above chance at all ages in OR, while OPR and OPCR memory emerged after 7th week for all three strains. The developmental trajectory of OCR memory was the most inconsistent with our previous analyses, with SD litters performing above chance levels after 5 weeks, LEH after 6 weeks and LH after 4 weeks of age.

Discussion

We investigated the ontogeny of episodic-like object-place-context memory and of memory in three tasks requiring memory for objects, object-context and object-place associations in rats. We found that three different outbred rat strains that are commonly used in basic neuroscientific and neuropsychiatric research exhibit remarkably similar developmental trajectories in their ability to recognize objects and object-context, object-place and object-place-context associations. Moreover, the trajectories for the different tasks are distinct. This likely reflects the development of the distinct neural circuits needed to support encoding and/or retrieval of memory in the different tasks. Interestingly, the developmental trajectories were unaffected by study design (i.e., longitudinal or cross-sectional), suggesting that repeated exposure to objects and contexts did not affect the developmental trajectory of object, object-context, object-place or object-place-context memory in the current study. This work adds to a large body of literature on the developmental trajectories of cognition in rodents (Hunt et al., 2016; Tan et al., 2017; Donato et al., 2021).

Here we report that rats were able to recognize objects from the first testing time point (3–4 weeks old depending on strain) in our studies (**Figures 2**, **6**). This is consistent with our previous work and findings from other labs showing that rats exhibit object memory as early as two-weeks old (Ainge and Langston, 2012; Krüger et al., 2012; Jablonski et al., 2013; Westbrook et al., 2014; Cruz-Sanchez et al., 2020). The perirhinal cortex is generally agreed to be required for object recognition memory whereas the entorhinal cortex, hippocampus (HPC), and medial prefrontal cortex (mPFC) are not (although there is debate concerning the role of the HPC at longer retention intervals and its normal role in the intact brain) (Dix and Aggleton, 1999; Brown and Aggleton, 2001; Eacott and Norman, 2004; Norman and Eacott, 2005; Langston et al., 2010; Wilson et al., 2013a,b; Chao et al., 2016). Although, the postnatal development of rat perirhinal cortex has not been fully characterized, morphological analysis of rat perirhinal neurons between birth and late adolescence (P45) suggests that perirhinal cortex may be fully developed around the time of eye-opening (P12–15) (Furtak et al., 2007). This would be consistent with OR memory (at least for relatively short retention intervals) developing at around this age. Overall, the demonstration that object recognition memory is established early in juvenile development is fundamental for the interpretation of findings from more complex types of memory involving associations between objects and spatio-temporal features of the environment. Being able to remember object identities at all experimental time points suggests that the different developmental trajectories observed for the other three tasks are not due to inability to distinguish novel from familiar objects.

In contrast to object memory, the ability to recognise object-context associations did not emerge until around 5 weeks of age in all three rat strains (**Figures 3**, **6**). Our data appear to be at odds with previous work from Ramsaran et al. (2016a,b), who showed that object-context recognition memory emerges during the second week of life in LEH rats (Ramsaran et al., 2016b). However, there are some crucial methodological differences between our work and that of Ramsaran et al. (2016b). The differences are centered on the nature of the contextual information. When contexts differ in testing arena wall and floor colour and texture as well as in polarising intra-maze cues, and the arenas are in different experimental rooms providing different distal spatial cues, rats can detect novel object-context association from two weeks old (Ramsaran et al., 2016b). If the two contexts do not differ in local contextual information but the two testing arenas are situated in different experimental rooms with different distal spatial information, OCR memory emerges during the third postnatal week (Ramsaran et al., 2016b). In our experiments, contexts were defined only by floor and wall inserts with different texture and color, while intra-maze and distal cues conferring polarising spatial information, as well as the position of the arena within the room, remained the same between testing phases. Therefore, the contextual differences in our experiments are very different than those in the Ramsaran et al studies. It is plausible that the nature of the contextual differences (i.e., intra-maze contextual information, prominent directional cues and spatial frame, spatial frame only, intra-maze only) may be a key determinant of the neural circuits that are required to support OCR memory. The differing developmental trajectories observed may therefore

reflect different development of these circuits. Alternatively, the differences between the studies could be explained by the fact that in the studies by Ramsaran et al. (2016a,b), rats were tested only once in a single task (OCR), whereas the rats in our study had been tested on an OR task earlier on the same day. Therefore, object or context interference from OR testing in the same day could have affected the ontogeny of OCR memory in our study.

Using a very similar protocol to one used in our studies, where context is based only on local contextual cues (floor and walls) but all distal and polarising spatial information is kept constant, it has been demonstrated that the lateral entorhinal cortex (LEC) is necessary for object-context memory (Wilson et al., 2013a,b), as is postrhinal cortex (Norman and Eacott, 2005), but not HPC (Langston and Wood, 2009), or fan cell inputs from LEC to HPC (Vandrey et al., 2020). However, when contextual differences involve more salient changes in spatial frames (e.g., different testing room, different distal cues, or different arena geometry and polarising intra-maze cues) then HPC is necessary (Balderas et al., 2008; Barker and Warburton, 2020). In addition to postrhinal cortex, LEC and HPC, the medial entorhinal cortex (MEC) could also be involved in object-context associative memory, as it has been shown to be essential for detection of contextual novelty such as texture and colour in floor and wall of an enclosure as well as convey contextual information to the hippocampus (Hunsaker et al., 2013; Kitamura et al., 2015).

Taken together, the present evidence suggests that postrhinal cortex, LEC, and possibly MEC (but not HPC) play a key role in associating contexts with objects when the contexts can be discriminated only on the basis of local non-spatial intra-maze cues. This leads to the hypothesis that this circuitry may not develop sufficiently to support OC memory until around five weeks of age. While the postnatal development of LEC is largely unknown, *in vivo* electrophysiological studies by us and others suggest that the functional maturation of spatial firing of MEC neurons may be complete by around five weeks, consistent with this time frame for OCR emergence (Langston et al., 2010; Wills et al., 2010). In contrast, when contexts can be discriminated on the basis of distal spatial information and/or geometric/polarising changes to the environment, then the hippocampus and its interactions with MEC may play a more prominent role in object-context memory (Shan et al., 2022). Consistent with this proposal, the spatial information contained in place cell firing as well as the stability of spatial representations between exposures in the same environment are similar to adult levels by four weeks of age (Langston et al., 2010; Wills et al., 2010; Farooq and Dragoi, 2019).

Memory in the OPR task requires the binding of location within the environment and object identity information and is known to depend on the coordination of a number of intact brain circuits, including the LEC and its connections to mPFC (Wilson et al., 2013b; Chao et al., 2016). Memory in this version of the task does not require the hippocampus, at least at the short retention intervals used in the current study (Langston and Wood, 2009). We suggest that the functional maturation of LEC-mPFC circuits may be dictating the developmental trajectory of OPR as explained in our discussion of OPCR memory below. The trajectory we observed in the OPR task differs from that previously reported for the more commonly used Object Location (OL) task, which tests the ability to detect that an object has moved to a novel location rather than memory for associations between specific objects and their locations. The ability to detect spatial novelty in the OL task has been reported to be in place by three weeks old (Krüger et al., 2012; Jablonski et al., 2013). As OL recognition does not require LEC or mPFC (Chao et al., 2016) but instead requires an intact hippocampus (Barker and Warburton, 2011), the developmental emergence of OL memory is consistent with the early development of the hippocampus described above.

The late emergence of OPR memory in the current study contradicts our previous findings that OPR memory is in place shortly after 4 weeks of age (Ainge and Langston, 2012). At face value this is an unexplained result. However, our previous work involved cross-sectional testing in only OR and OPR memory tasks which took place in a single context. Therefore, rats were not exposed to multiple contexts during habituation and during OCR testing before OPR. It is plausible that the current experimental design using 2 contexts leads to more memory interference during any two-day testing time point.

The late development of episodic-like OPCR memory in rats is generally consistent with the developmental trajectory of episodic memory in humans (Guillery-Girard et al., 2013; Riggins et al., 2020; Ngo et al., 2021). OPCR memory requires an intact HPC and LEC as well as LEC-HPC, LEC-mPFC, and HPC-PFC interactions (Langston and Wood, 2009; Wilson et al., 2013b; Chao et al., 2016; Barker and Warburton, 2020; Vandrey et al., 2020). Given that OPR and OPCR appear to have a very similar developmental trajectory, which is distinct from the LEC-dependent OCR trajectory, it is unlikely that LEC circuit maturation controls the emergence of OPR and OPCR memory (**Figures 4**, **5**, **7**).

We propose that the late emergence of both OPCR and OPR memory may be dictated by the time course of mPFC circuit maturation. This developmental trajectory is consistent with previous work showing that young adolescent rats (<P39) are worse at attentional set-shifting and are more impulsive compared to young adults (>P66) (Newman and McGaughy, 2011; Doremus-Fitzwater et al., 2012). Both impulsivity and attention set-shifting behaviour have been linked to prefrontal function (Birrell and Brown, 2000; Bradshaw et al., 2016).

While several aspects of mPFC circuit function develop early in postnatal development (Chini and Hanganu-Opatz, 2021) others mature much later during adolescence in rats (Caballero et al., 2016) and even during adulthood in mice (Mukherjee et al., 2019). More specifically, local inhibitory networks within

mPFC that allow gating of HPC inputs do not develop fully until the seventh postnatal week in rats (Tseng and O'Donnell, 2007; Caballero et al., 2016; Caballero and Tseng, 2016). While the developmental trajectory of LEC-mPFC interactions is not known, it is plausible that the protracted development of mPFC inhibition determines the functionality of both LEC-mPFC and HPC-mPFC interactions and ultimately the developmental trajectory of OPR and OPCR memory.

A previous study has reported that object-place-context recognition memory is in place during the fifth postnatal week (Ramsaran et al., 2016a) which appears to contradict our current findings. However, as discussed above, the contexts used in the experiments by Ramsaran et al. (2016a) consisted of radically different testing enclosures situated in different experimental rooms, which may be coded by MEC-HPC. Therefore, OPCR memory in which contexts can be defined on the basis of distal spatial information may rely on different circuits that OPCR memory that requires binding of object and place information with non-polarising intra-maze contextual cues. We suggest that the LEC and its interactions with both HPC and mPFC may only be required in the latter case. An alternative explanation for the different findings between our study and Ramsaran et al., with regards to the age of emergence of OPCR memory, may be that rats in our study were tested on OR, OCR, and OPR prior to OPCR, leading to memory and contextual interference. Future experiments in which rats are tested in a single task (either OCR, OPR, or OPCR) at just one time point will allow us to test whether previous testing within each time-point leads to interference, thereby delaying the ontogeny of these abilities.

The current findings allow us to formulate two testable hypotheses. The first is that local LEC circuit function matures around the fifth postnatal week to support object-context recognition memory. The second is that mPFC circuit function, more specifically the inhibitory control of inputs from both LEC and HPC, matures around the seventh postnatal week to support object-place and object-place-context memory. One advantage of rat models compared to mice is the ability to use *in vivo* electrophysiology to study circuit function during juvenile development (Langston et al., 2010; Wills et al., 2010; Farooq and Dragoi, 2019). Therefore, an obvious future direction from our findings is to explore the developmental trajectory of circuit functions in relation to the developmental trajectories of object-context, object-place and episodic-like object-place-context memory.

The developmental trajectories we report here raise questions about the ontogeny of other associative object recognition memory tasks that assess aspects of episodic-like memory. For example the what-where-when (WWWhen) task that requires subjects to associate object identity, object location and temporal order/recency of object exposure (Kart-Teke et al., 2006). Similar to OPC (WWWhich) memory, WWWhen memory requires HPC and mPFC-LEC interactions (Chao et al., 2016; Drieskens et al., 2017; de Souza et al., 2019). Interestingly though, WWWhen and WWWhich memory have been shown to be differently affected by normal ageing and neurodegeneration (Davis et al., 2013), suggesting that the neural circuits mediating these tasks may differ in some way. It would be interesting to test whether the developmental trajectory of WWWhen and the WWWhich (OPCR) memory follow similar developmental trajectories.

Despite the overwhelming similarities in the developmental trajectories of the three rat strains in our study, there are some small differences, with the most usual being in the amount of exploration different strains exhibited in sample or/and test phase. This could reflect known differences in vision between albino and pigmented rat strains (Andrews et al., 1995; Prusky et al., 2002; Kumar et al., 2015; Waite et al., 2021). Despite the differences in object exploration between rat strains, our correlation analyses did not reveal any consistent relationships. The occasional significant correlations between total object exploration in the sample or test phases and discrimination performance varied between being negative and positive, and were strain specific (**Supplementary Table 3**). It has previously been argued that ensuring a minimum amount of object exploration during sampling phase is important for the interpretation of discrimination performance data (Cohen and Stackman, 2015). In our studies rats exhibited high levels of sample phase exploration throughout age points and tasks. Collectively, these data suggest that the developmental trajectories described here are not secondary to fluctuations in sample (or test) phase object exploration behaviour.

While our study was not designed to address the importance of sex as a determinant of object recognition memory development, we were able to explore this question in our cross-sectional experiment with LH rats. The absence of sex-dependent developmental trajectories in LH rats is consistent with recent research and meta-analyses suggesting that sex is not a significant determinant of object memory performance (Becker et al., 2016; Becegato and Silva, 2022).

Shared genetics and maternal environment in multiparous species can lead to high similarity between outcome variables in littermates that violate statistical independence. The most appropriate method to address intra-litter correlations and litter oversampling is considered to be the use of linear mixed effects models with litter included as a random effect (Golub and Sobin, 2020). Traditionally, the most stringent statistical approach has been to use one animal per litter or to average across animals from a litter, such that litter is the experimental unit. Here, both statistical analysis approaches for analysing the developmental trajectory of memory in SD and LEH rats have led to similar results.

Data from the cross-sectional study originated from rats coming from only 2–3 litters for each age group, with all animals from any given litter being assigned to a single age group. This experimental design poses some statistical challenges. Given the known intra-litter statistical dependencies, we have

inadvertently oversampled from each litter. Taking advantage of the fact that we used both female and male rats in this study, we fitted linear mixed effects models with sex-within-litter as a nested random effect. This approach, similar to the one used in the longitudinal studies, can begin to account for intra-litter statistical dependencies. Our supplemental analyses on data averaged across rats of the same sex from the same litter (sex-within-litter as statistical unit), while underpowered, yielded very similar results to our main analyses.

An alternative approach for the cross-sectional study would have been to assign different littermates to different age groups, resulting in a quasi-repeated study design (i.e., same litter across multiple age groups). However, this approach presents its own unique limitations. The different duration between weaning and testing for littermates can yield distinct experiential contributions to behaviour, and complicate the effects of intra-litter correlations. Therefore, sampling for cross-sectional studies is particularly challenging when attempting to reduce the number of experimental subjects used.

Taken these considerations into account, and the fact that the developmental trajectories of OR, OCR, OPR, and OPCR memory in SD, LEH, and LH each mirror other, we suggest that it is extremely unlikely that the developmental trajectories we report here can be accounted for by our sampling methods.

Genetic rat models of neurodevelopmental conditions are providing new insights into behavioural and circuit abnormalities associated with mutations in genes of interest (Till et al., 2015; Asiminas et al., 2019; Berg et al., 2021). In order to understand the neural and circuit pathophysiology associated with cognitive deficits that emerge during postnatal development, and to identify key time points and targets for therapeutic intervention, it is important to utilize these rat models (Asiminas et al., 2019). More specifically, episodic-like memory tasks can offer good face validity since episodic memory is affected in neurodevelopmental conditions such autism and schizophrenia (Wang et al., 2010; Ragland et al., 2015; Cooper and Simons, 2019). The differential development of different types of associative recognition memory offers a unique opportunity to delineate the developmental trajectory and function of neural circuits supporting the different components of episodic memory. Using spontaneous object exploration tasks to explore deviations from normal developmental trajectories in specific tasks can provide a window to the circuit pathophysiology and progression of neurodevelopmental conditions.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Ethics statement

This animal study was reviewed and approved by University of Edinburgh and University of Dundee Animal Welfare and Ethical Review Boards.

Author contributions

AA, SL, RL, and EW conceived and designed the experiments. AA and SL collected and analysed the data. AA wrote the first draft manuscript. AA, RL, and EW interpreted all the results and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.969871/full#supplementary-material

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Infusing zeta inhibitory peptide into the perirhinal cortex of rats abolishes long-term object recognition memory without affecting novel object location recognition

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Infusing the amnesic agent zeta inhibitory peptide (ZIP) into the dorsal hippocampus disrupts established long-term object location recognition memory without affecting object identity recognition, which likely depends on the perirhinal cortex. Here, we tested whether infusing ZIP into the perirhinal cortex can abolish long-term memory supporting object identity recognition, leaving long-term object location recognition memory intact. We infused ZIP into the perirhinal cortex of rats either 1 day or 6 days after exposing them to two identical objects in an open field arena. One day after ZIP infusion, that is, 2 or 7 days after object exposure, we either assessed whether the animals recognized that now one of the two objects was novel or whether they recognized that one of the two familiar objects was at a new location. Our results show for both retention intervals, infusions of ZIP into the perirhinal cortex impaired novel object recognition but spared novel object location recognition. Rats that received a scrambled version of ZIP had no deficit in either test at both retention intervals and expressed stronger novel object recognition compared to rats infused with ZIP. These findings support the view that object recognition depends on dissociable memory representations distributed across different brain areas, with perirhinal cortex maintaining long-term memory for what objects had been encountered, and hippocampus supporting memory for where these objects had been placed.

KEYWORDS

perirhinal cortex, object recognition, memory maintenance, long-term memory, PKMzeta inhibitors

Introduction

The question of which brain structures are essential for object recognition memory has been controversially debated because results from humans, non-human primates, and rodents have not always been in agreement (Brown and Banks, 2014; Cohen and Stackman, 2015). The overwhelming evidence seems to identify the perirhinal cortex as the area required for hosting long-term memory representations supporting object recognition (Winters and Bussey, 2005; Winters et al., 2008). It is less clear whether the hippocampus also supports object recognition memory, as some studies indicate its involvement in object recognition tasks, and some studies suggesting otherwise (Warburton and Brown, 2015). While these variations sometimes depend on specific task conditions, they may also arise from differences in the methods used to interfere with processes in the hippocampus. For example, lesions and pharmacological interventions have led to different conclusions regarding the role of the hippocampus in object recognition memory (Broadbent et al., 2004; Brown et al., 2010; Cohen and Stackman, 2015).

This complex situation may benefit from an approach that targets established memory, after memory formation and consolidation have concluded, outside the context of other memory processing phases such as retrieval and reactivation. This can be achieved with the amnesic agent zeta inhibitory peptide (ZIP), which has been designed to transiently block the activity of protein kinase M zeta (PKMζ), an autonomously active protein kinase C (PKC) isoform. Several lines of evidence suggest that PKMζ contributes fundamentally to various forms long-term memory. For example, infusing ZIP into the hippocampus can impair consolidated spatial memories (Pastalkova et al., 2006; Serrano et al., 2008; Migues et al., 2010), and infusing it into the basolateral nucleus of the amygdala can disrupt long-term auditory (Migues et al., 2010) as well as contextual fear memory (Serrano et al., 2008). We have previously shown that infusing ZIP into the dorsal hippocampus disrupts long-term memory supporting the recognition of novel object locations, without affecting the ability to recognize novel objects (Hardt et al., 2010), which likely depends on perirhinal cortex.

To complement these results, here we tested whether infusing ZIP into the perirhinal cortex—at time points when it cannot affect memory formation or retrieval—impairs novel object recognition while sparing novel object location recognition.

Methods

Animals

We obtained male Long-Evans rats at 250–300 g from Charles River, Canada, and housed them in pairs with environmental enrichment (wooden gnawing block, PVC tube) in transparent plastic cages. Rats consumed food and water *ad libitum*. The light in the animal colony went on at 7 A.M. and off at 7 P.M. We performed our experiments between 9 A.M. and 2 P.M. All procedures followed the relevant guidelines published by the Canadian Council on Animal Care, and the Faculty Animal Care Committee at McGill University reviewed and approved them.

Surgery

Surgeries followed the procedures used in our earlier experiment testing the role of PKMζ in object location memory in the dorsal hippocampus (Hardt et al., 2009, 2010). We gave rats intraperitoneal injections of an anesthetic mixture consisting of xylazine (3.33 mg/ml), ketamine (55.55 mg/ml), and Domitor (0.27 mg/ml) in a volume of 1 ml/kg. Once animals were in deep anesthesia, we shaved their heads and then placed them into a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). A midline incision exposed the skull, and we implanted three jeweler screws and two guide cannulas (22 gauge, P1 Technologies, Roanoke, VA, USA) into each hemisphere aiming at the perirhinal cortex (pRh) at coordinates A/P -5.5 mm, M/L 6.6 mm, D/V -6.7 mm (Paxinos and Watson, 2004). We applied dental cement to stabilize the cannulas and inserted obturators to prevent blocking and contamination. Thirty minutes before the end of surgery, we injected subcutaneously the analgesic Carprofen. We reversed anesthesia with an intraperitoneal injection of Antisedan (7.5 mg/kg). We let rats recover from surgery for 7 days, during which we handled them and regularly cleaned the obturators with 70% ethanol in sterile water.

Drug infusions

Zeta inhibitory peptide (Myr-SIYRRGARRWRKL-OH_2, Anaspec) or scrambled ZIP (scrZIP); (Myr-RLYRKRIWRSAGR-OH; Anaspec) were dissolved in 100 mM Tris-saline to a final concentration of 10 mM and the pH was adjusted to 7.2. We infused 1 μl (10 nmol) of the peptide solution bilaterally into the perirhinal cortex using 28-gauge microinjectors (P1 Technologies, Roanoke, VA, USA), connected with polyethylene tubing to a Hamilton syringe, at a speed of 0.25 μl/min (i.e., a total volume of 1 μl in 4 min). After the infusion, we left the microinjectors in place for 90 s to allow drugs to diffuse away from the injector tip. Between animals, we cleaned the microinjectors with 70% ethanol in sterile water and thoroughly dried them with paper towels.

Apparatus

We used an open field measuring 60 × 60 × 60 cm, made of laminated particle board, placed onto a wooden platform 10 cm

above the floor. A digital camera positioned 130 cm above the field recorded each trial. The open field was in the center of a room measuring 3 x 3 m, having no windows and one red door. There were no other salient distal cues in the room. The indirect lighting produced 15 lux, measured at the floor of the open field. The floor of the open field was covered with about 4 cm of the same type of sawdust bedding also used for the home cages. We changed the bedding in the open field between experiments. As stimuli, we used several everyday (junk) objects with no known biological significance for the rats. The objects were glued to the bottom of mason jars, and the top of the jars was then secured by screwing the jars into jar lids, which were fastened to the floor with screws and wing nuts (Hardt et al., 2010; Migues et al., 2010, 2014). To keep track of each copy of an object, we wrote a number onto the rim of the mason jar to which it was glued, so that rats were not able to see these identifying marks.

Behavioral procedures

We ran two studies consisting of two experiments each; one study tested memory after a 2 days memory retention interval, the other one tested memory after a 7 days interval. For each study, we used a new group of rats. Each study had two experiments and the rats used for a study took part in both. In each study, one experiment tested for object identity recognition memory, the other one for object location recognition memory. There were between 10 and 14 days between each experiment, and their order (i.e., whether we assessed novel object or novel location recognition first) was counterbalanced. We neither used objects nor locations twice for any given rat in the two studies, and we used a different open field in a different room for each study.

All experiments had four phases—Habituation, Sampling (i.e., training), Drug Infusion, and Probe (i.e., memory test). The procedures used in the experiments closely followed those we used in our earlier study (Hardt et al., 2010).

Habituation

About 7 days after surgery, we habituated the rats to the open field over four consecutive days. Each day, we placed rats for 10 min into the open field. The open field contained two identical copies of the same object during all 4 days of Habituation, but the position of the two objects changed from day to day. We put objects always in opposing corners (i.e., NE-SW or NW-SE). We lowered rats into the open field with their head facing an empty corner. Between rats, we removed the objects, cleaned them with 70% ethanol in distilled water, removed feces from the arena, and swirled the arena floor bedding around to disperse any possible odor markings left behind.

Sampling

One day after the last Habituation trial, we presented animals with two copies of an object that they had not seen before. We placed the objects into opposing corners and they stayed at the same position throughout all Sampling trials. For Exps 1 and 2, we trained rats for two consecutive days, twice each day, with one session in the A.M., another one about 4–5 h later in the P.M. For Exps 3 and 4, we trained them for seven consecutive days, 10 min per day, during the A.M. phase of the day. Sampling trials were always 10 min long. We lowered rats into the open field, as during Habituation, facing a corner that had no object.

Infusions

For Exps 1 and 2, rats received one infusion the day after the last sampling session. For Exps 3 and 4, rats received the infusion 6 days after the last sampling session. Infusions occurred between 11 A.M. and 1 P.M. in the home colony of the animals.

Probe

One day after the infusion, i.e., 2 days after Sampling in the first study (Exps 1 and 2), and 7 days after Sampling in the second study (Exps 3 and 4), we tested long-term recognition memory. To assess object identity recognition (Exps 1 and 3), we presented rats with another copy of the object used during Sampling and one novel object, both placed where objects had been before. Novel and familiar objects were counterbalanced across conditions. To assess location novelty recognition (Exps 2 and 4), we presented rats with the same objects used during Sampling but moved one of them to a novel location. In each Probe trial, we lowered rats into the open field facing a corner that did not contain an object, and that was furthest away from both objects (the latter only relevant for object location recognition tests). Each Probe trial took 3 min.

Histology

We deeply anaesthetized animals and then decapitated them. We removed the brains and fixed them in a mixture of 4% paraformaldehyde and 30% sucrose–saline. We used a cryostat to obtain sections of 50 μm thickness. We verified the placement of the implanted cannulas with a light microscope. We included animals in the analyses when an experimenter blind to the treatment group detected the injector tips inside perirhinal cortex in both hemispheres (**Figure 1**).

Data analysis

We manually scored the recorded videos. We considered rats exploring objects when they directed their nose at an

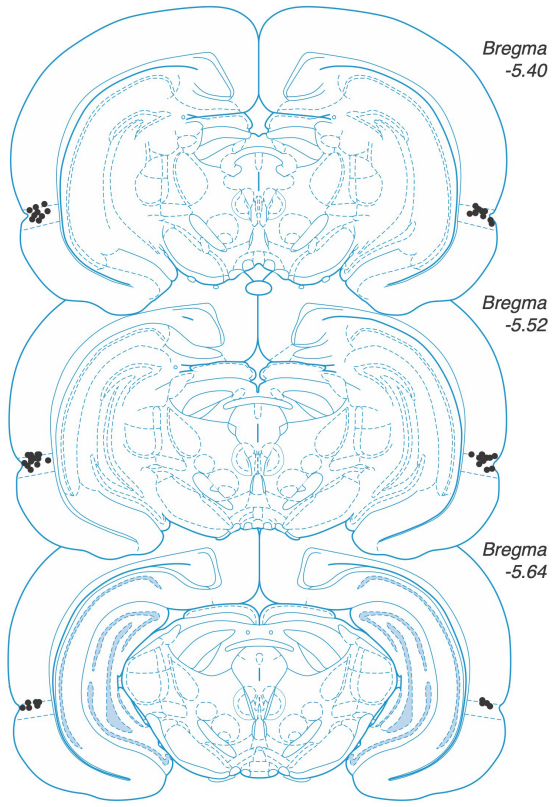

FIGURE 1
Placement of microinjector. tips in perirhinal cortex. Placements shown on coronal sections from rat brain atlas by Paxinos and Watson (2004).

object at an angle of at least 45 degrees and no farther away than 2 cm. We did not consider sitting and climbing onto objects as exploratory behavior (Ennaceur and Delacour, 1988). We scored the videos and then pre-processed the data with in-house software prior to statistical analyses. To determine novelty preference (*d*), we measured the total amount of time rats spent exploring the novelty (*t_new*; object or location) and the familiarity (*t_old*), and calculated *d* as $d = (t\_new - t\_old)/(t\_new + t\_old)$. The novelty preference ratio *d* can take any value between -1.0 and 1.0, with $d = 0$ denoting equal exploration of both novelty and familiarity, i.e., the absence of exploratory preferences and suggesting that animals do not express memory for the object locations or object identity, respectively, from the training phase; values of *d* significantly higher than 0 indicate that rats express memory. We used Jamovi (version 2.3)[1] for our statistical analyses. All data were normally distributed, and therefore we used *t*-tests or repeated-measures ANOVAs to determine significant group differences and one-sample *t*-tests to compare *d* against 0 to determine whether groups expressed memory. The threshold for accepting the null hypothesis was set to alpha = 0.05. For significant effects, we report the effect size measures partial eta squared (η^2_p), or Cohen's d.

1 https://www.jamovi.org

Results

Infusing zeta inhibitory peptide into the perirhinal cortex impairs recent (1 d old) long-term object but not object location recognition memory

We exposed rats for 2 days to two identical copies of an object in our test arena (**Figure 2A**), and infused ZIP or the scrambled version scrZIP into the perirhinal cortex 24 h later. One day after the infusion into perirhinal cortex, we tested half the rats of each drug infusion group for their memory of what object had been presented (object identity) or memory for where the objects had been placed (location memory). Between 10 and 14 days after the memory test, we repeated the experiment, infusing the same drug into the same rats, testing them for the memory type we had not assessed before (i.e., location or identity, respectively). A repeated-measures ANOVA on novelty preference (*d)* with memory type (identity vs. location) as the repeated factor and treatment (scrZIP vs. ZIP) as between-subjects factor revealed a significant interaction, $F(1,10) = 7.0$, $p = 0.02$, $\eta^2_p = 0.41$, but no significant main effect of memory type, $F < 1$, or treatment, $F(1,10) = 3.7$, $p = 0.08$. *Post-hoc* tests (Tukey) determined that rats infused with ZIP expressed a significantly lower preference to explore the novel object compared to rats that received scrZIP, $t = -3.2$, $p < 0.04$; no other comparison was significant. One sample *t*-tests comparing novelty discrimination against what would be expected by chance alone (i.e., zero) determined that that rats infused with ZIP, $t(5) = 5.0$, $p = 0.004$, Cohen's d = 2.0, and rats infused with scrZIP, $t(5) = 2.7$, $p = 0.04$, Cohen's d = 1.1, preferred to explore the object at the novel location; however, only rats infused with scrZIP also significantly preferred to explore the novel object, $t(5) = 5.5$, $p = 0.003$, Cohen's d = 2.3 [ZIP group: $t(5) = 1.4$, $p = 0.23$]. A repeated-measures ANOVA on exploratory activity with memory type (identity vs. location) as the repeated factor and treatment (ZIP vs. scrZIP) as between-subjects factor revealed no significant effects (memory type: $F < 1$; treatment: $F(1,10) = 2.4$, $p = 0.15$; interaction: $F < 1$). Taken together, these results suggest that infusing ZIP into the perirhinal cortex can impair 2 days old recognition memory for "what" objects had been encountered, leaving intact long-term recognition memory for "where" objects had been placed. The absence of differences in exploratory activity between the groups in each memory test indicates that differences in motivation or

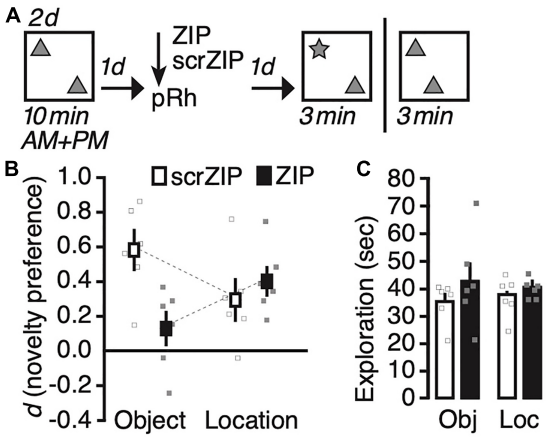

FIGURE 2
Infusing zeta inhibitory peptide (ZIP) into the perirhinal cortex impairs recent (1 day old) long-term object recognition memory but not object location recognition memory. **(A)** Experimental protocol. We trained rats twice per day on two consecutive days, exposing them for 10 min to two copies of an object in an open field arena. One day later, we infused ZIP (10 nmol) or scrZIP (10 nmol) into the perirhinal cortex. The following day, we put animals back into the open field. For half of the rats, we replaced one familiar object with a novel one (assessing novel object recognition), for the other half, we moved one object to a different location (assessing novel object location recognition). Rats took part in both assessments, with about 10–14 days between experiments (i.e., between memory test and habituation). **(B)** Infusing ZIP, but not scrambled ZIP (scrZIP) into the perirhinal cortex impairs the expression of object recognition memory, but not object location memory. **(C)** Exploratory activity during the test was the same for both groups in each assessment. Error bars ± 1 standard error of the mean.

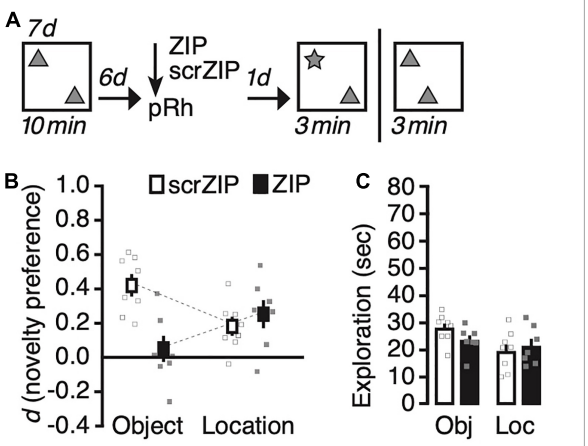

FIGURE 3
Infusing zeta inhibitory peptide (ZIP) into the perirhinal cortex impairs remote (6 days old) long-term object recognition memory but not object location recognition memory. **(A)** Experimental protocol. We trained animals for seven consecutive days, exposing them 10 min per day to two copies of the same object in an open field arena. Six days later, we infused ZIP (10 nmol) or scrZIP (10 nmol) into the perirhinal cortex. The following day, we assessed memory and repeated the experiment memory as before (**Figure 2**). **(B)** Infusing ZIP into the perirhinal cortex impairs novel object recognition, but not novel location recognition. **(C)** Rats explored objects more in tests in which a novel object was present than when a familiar object moved to a new location, irrespective of whether they receive ZIP or scrZIP. Error bars ± 1 standard error of the mean.

motility cannot account for the pattern of novelty preference during the tests.

Infusing zeta inhibitory peptide into the perirhinal cortex impairs remote (7 days old) long-term object but not object location recognition memory

We next explored whether ZIP will impair novel object recognition for more remote long-term memories. We changed the training procedure to promote the formation of location memory that lasts for at least 7 days (Migues et al., 2016), and adjusted the retention interval accordingly (**Figure 3A**). During Sampling, we exposed rats to two copies of an identical object—the same we used for the first study—daily for 10 min for seven consecutive days, then infused ZIP or scrZIP into the perirhinal cortex 6 days after the last training session; finally, we tested recognition memory the following day, 7 days after the last Sampling trial. As before, we tested half the rats on object location recognition memory and half on object identity recognition memory, and repeated the experiment between 10–14 days later, as described above, then testing rats for the memory we had not assessed already. A repeated-measures ANOVA on novelty preference (d) with memory test (identity vs. location) as the repeated factor and treatment (scrZIP vs. ZIP) as the between-subjects factor detected a significant interaction, $F(1,13) = 17.8$, $p = 0.001$, $\eta^2_p = 0.58$, no significant main effect of test, $F < 1$, nor a significant main effect of treatment, $F(1,13) = 4.2$, $p = 0.06$, which, however, approached significance and suggested that novelty preference in general tended to be stronger in animals that received infusions of scrZIP into the perirhinal cortex, as compared to rats that received ZIP (**Figure 3B**). *Post-hoc* tests (Tukey) to further analyze the significant interaction revealed that rats receiving scrZIP preferred to explore the novel object significantly more so than animals that received ZIP, $t = 4.1$, $p = 0.007$. Also, novelty preference was significantly stronger for novel objects than for novel object locations in animals that received scrZIP, $t = 3.3$, $p = 0.029$. No other comparison was significant. One-sample t-tests comparing novelty preference against zero detected that rats infused with scrZIP and ZIP both preferred to explore the object at the novel location [scrZIP: $t(7) = 3.6$, $p = 0.009$, Cohen's d = 1.3; ZIP: $t(6) = 3.3$, $p = 0.016$, Cohen's d = 1.3], but that only rats infused with scrZIP also preferred to explore the novel object, $t(7) = 7.2$, $p < 0.001$, Cohen's d = 2.6 (ZIP:

$t < 1$). A repeated-measures ANOVA on exploratory activity with memory test (identity vs. location) and treatment (scrZIP vs. ZIP) as the between-subjects factor detected a significant main effect of memory test, $F(1,13) = 6.6$, $p = 0.023$, $\eta^2_p = 0.34$, no significant main effect of treatment, $F < 1$, and no significant interaction, $F(1,13) = 1.9$, $p = 0.19$ (**Figure 3C**). These results suggest that 6 d after acquisition, infusing ZIP into the perirhinal cortex can impair long-term memory for object identity, an intervention that does not impair the ability to recognize novel locations of objects. Our findings further imply that at this time point, situations in which rats encounter novel objects provoke more exploratory activity than situations in which a familiar object occupies a new location, irrespective of whether animals received ZIP or scrZIP into perirhinal cortex.

Discussion

We tested whether infusing ZIP into the perirhinal cortex of rats can disrupt long-term memory that supports identification of novel objects and novel object locations in a standard object recognition paradigm. We found that this intervention impairs the expression of recognition memory for novel objects, but not novel object locations, for both recent (1 day old) as well as remote (6 days old) memories. We thus replicate the findings of Outram et al. (2022), lending strong support to our respective results. Together with our earlier demonstration that infusing ZIP into the dorsal hippocampus disrupts long-term object location but not object identity memory, these data show that long-term memory for *what* has been encountered requires representations in perirhinal cortex, while memory for *where* things had been placed requires representations in dorsal hippocampus (Winters, 2004; Forwood et al., 2005).

Although our results add to the well-established position that perirhinal cortex critically supports object recognition, it seems likely that the hippocampus is involved in object recognition memory during different memory processing phases, or memory states, as well. For example, lesions to the hippocampus impair, but not abolish, novel object recognition, in that sham-operated rats show a stronger novel object preference than rats who had received lesions to the hippocampus (Broadbent et al., 2009). Furthermore, under certain conditions, impairing dorsal hippocampal function can disrupt long-term object recognition memory. For instance, when the environment in which rats originally acquired object memory is modified when rats are briefly re-exposed to the objects they had encountered there earlier, subsequent infusions of the protein-synthesis inhibitor anisomycin into the dorsal hippocampus impair novel object recognition in a later memory test; absent changes to the context, this reactivation treatment leaves object recognition memory intact (Winters et al., 2011). Similar findings have been reported for interventions that disrupt the activity of PKMζ. For example, blocking PKMζ with ZIP or antisense in the dorsal hippocampus does not affect long-term novel object recognition memory unless these memories have been retrieved, or reactivated (Rossato et al., 2019). Recent findings in mice further suggest that the extent to which animals explore objects during the initial encounter moderates whether hippocampus or perirhinal cortex critically support long-term novel object recognition, such that longer exploration times engage the hippocampus, while shorter times recruit the perirhinal cortex (Cinalli et al., 2020). These exemplary findings suggest that in the normal brain, although the perirhinal cortex hosts memory representations necessary for novel object recognition, other brain areas, under certain conditions or during certain memory phases, such as acquisition, expression, and updating, also can critically contribute to the expression of novel object recognition or the processing of memory representations underpinning the recognition of novel objects. Thus, affecting interactions of these brain areas during certain mnemonic processing periods may result in acute or long-lasting modulation of the ability to recognize objects as being novel.

We used ZIP in our experiments because it has been widely shown to impair memory maintenance in a variety of tasks and animal models (Patel and Zamani, 2021). Several studies support the notion that ZIP disrupts long-term memory because it blocks the activity of PKMζ, promoting the internalization of GluA2-containing AMPA receptors (GluA2/AMPARs) from post-synaptic densities, thus rapidly reducing synaptic potentiation induced by learning and memory formation (Migues et al., 2010, 2014; Dong et al., 2015). It should be noted that whether PKMζ is the essential element of this maintenance processes, or whether other PKC isoforms are also recruited (Ren et al., 2013) has been controversially discussed and remains to be fully resolved (Cai et al., 2011; Kwapis and Helmstetter, 2013; Lee et al., 2013; Volk et al., 2013; Tsokas et al., 2016; Wang et al., 2016).

Irrespective of the mode of action, ZIP has the advantage that it can be administered at times when it unlikely affects other processes that could account for memory loss, such as acquisition, formation, expression, and the like, such that memory deficits can be attributed to impaired maintenance of long-term memory. There are some findings, however, that suggest that the effects of ZIP on memory retention may not arise from the assumed interaction with kinases relevant for memory maintenance, but from excitotoxic effects causing cell death (Sadeh et al., 2015), or from attenuating neural activity (LeBlancq et al., 2016). These alternative explanations could account for some of the amnesia observed with this peptide, but the results of other studies cast doubt on this

interpretation. First, several studies have shown that despite ZIP-induced memory loss, animals are able to learn and form new long-term memories, suggesting that neurotoxic effects cannot readily account for the retrograde amnesia following infusions of ZIP (Pastalkova et al., 2006; Sacktor, 2008; von Kraus et al., 2010). Second, the peptide GluA2-3Y that blocks the activity-dependent removal of GluA2/AMPARs (Lee et al., 2002; Scholz et al., 2010; Migues et al., 2016) prevents the amnesic effects of ZIP, as we have shown before (Migues et al., 2016) and (Outram et al., 2022) have replicated. If indeed ZIP acts mainly via inducing excitotoxic effects, then preventing the removal of AMPA receptors from post-synaptic membranes should not block the actions of ZIP. Finally, non-specific actions of ZIP cannot account for why infusing it into the perirhinal cortex affects novel object recognition, but not the oddity discrimination task in the study of Outram et al. (2022). In conclusion, future studies could exploit this peptide to dissect the role and contributions of various brain areas during different phases of object recognition tasks more carefully (Rossato et al., 2019).

Our study thus suggests that at least some of the processes maintaining long-term object recognition memory in the perirhinal cortex involve activity of PKMζ. Notably, impairing the activity of PKMζ impairs established long-term potentiation (LTP), but not long-term depression (LTD), and it is the latter form of synaptic plasticity that has been linked to object recognition memory (Warburton et al., 2003; Griffiths et al., 2008). For example, recording from perirhinal cortex in rats, (Zhu et al., 1995) found that the second exposure to an object resulted in changed responses in a subset of the recorded neurons, such that in perirhinal cortex 13% of neurons decreased their activity, while 9% increased it. By comparison, in the hippocampus, 3% of neurons decreased their response, while 9% increased it. These data suggest that object recognition memory seems to recruit mechanisms that dampen synaptic responses in the perirhinal cortex to a larger extent than the hippocampus, linking the former to processes found in long-term depression, i.e., synaptic weakening, more so than to processes found in long-term potentiation, i.e., synaptic strengthening. Long-term depression critically depends on the internalization of GluA2/AMPARs from presynaptic membranes (McCormack et al., 2006; Diering and Huganir, 2018), and, to study its role in object recognition memory Griffiths et al. (2008) targeted this process in perirhinal cortex. Using a lentiviral vector to express a peptide in perirhinal cortex that interferes with the binding of the clathrin adaptor protein AP2 and GluA2 – an event required for GluA2/AMPAR internalization – they impaired object recognition memory in rats. Because rats acquired object recognition memory while the peptide was being expressed, the outcomes of this study cannot address whether acquiring, maintaining, or expressing object recognition memory requires GluA2/AMPAR endocytosis, yet it suggests that LTD contributes to this type of memory.

Taken together, these data suggest that processes underpinning LTD also promote object recognition memory in perirhinal cortex. Our data, as well as the findings from Outram et al. (2022), however, indicate that forms of synaptic plasticity involved in LTP also are critical for object recognition memory in this brain region. Specifically, the results of Outram et al. (2022) show that the amnesic effects of ZIP on object recognition memory involve the internalization of GluA2/AMPARs, suggesting that maintaining long-lasting object recognition memory depends on forms of synaptic plasticity that are critical for LTP, but not LTD. Thus, while these and our results seem in conflict with earlier findings, they make sense from the position that memory reflects patterns of synaptic connectivity arising from adjusting synaptic weights, i.e., the strengthening and weakening of synaptic connections, requiring processes involved in LTP as well as those involved in LTD (Norman, 2010). Future studies could address how the interplay of various forms of synaptic plasticity supports the formation and maintenance of long-term object recognition memory in the perirhinal cortex.

In summary, our findings support the view that different brain areas support memory of *what* was encountered *where* that is assessed in novelty recognition tests. Clearly, when animals explore an environment they acquire, without externally provided reinforcement, complex memories about objects and their spatial relations, with the former involving perirhinal cortex and the latter hippocampus, among other brain areas. This distributed representational nature might help explain why disrupting hippocampal processing can impair newly acquired or reactivated object recognition memory (Winters et al., 2011). Our findings lend further support for this perspective, indicating that object recognition memory represents a mnemonic capacity that relies on interactions of various brain regions, notably prefrontal cortex, hippocampus, and perirhinal cortex (Bussey et al., 2005; Murray et al., 2007; Cowell et al., 2010; Saksida and Bussey, 2010; Warburton and Brown, 2015; Chao et al., 2020). As such, it presents a well-suited rodent paradigm to study regions and processes likely underpinning human episodic and semantic memory, as others have noted before.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Faculty Animal Care Committee of McGill University.

Author contributions

OH and PM designed study. OH and KA conducted behavioral studies and analyzed data. All authors wrote the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Spontaneous Object Exploration in a Recessive Gene Knockout Model of Parkinson's Disease: Development and Progression of Object Recognition Memory Deficits in Male *Pink1–/–* Rats

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Cognitive impairments appear at or before motor signs in about one third of patients with Parkinson's disease (PD) and have a cumulative prevalence of roughly 80% overall. These deficits exact an unrelenting toll on patients' quality and activities of daily life due in part to a lack of available treatments to ameliorate them. This study used three well-validated novel object recognition-based paradigms to explore the suitability of rats with knockout of the PTEN-induced putative kinase1 gene (*Pink1*) for investigating factors that induce cognitive decline in PD and for testing new ways to mitigate them. Longitudinal testing of rats from 3–9 months of age revealed significant impairments in male *Pink1–/–* rats compared to wild type controls in Novel Object Recognition, Novel Object Location and Object-in-Place tasks. Task-specific differences in the progression of object discrimination/memory deficits across age were also seen. Finally, testing using an elevated plus maze, a tapered balance beam and a grip strength gauge showed that in all cases recognition memory deficits preceded potentially confounding impacts of gene knockout on affect or motor function. Taken together, these findings suggest that knockout of the *Pink1* gene negatively impacts the brain circuits and/or neurochemical systems that support performance in object recognition tasks. Further investigations using *Pink1–/–* rats and object recognition memory tasks should provide new insights into the neural underpinnings of the visual recognition memory and visuospatial information processing deficits that are often seen in PD patients and accelerate the pace of discovery of better ways to treat them.

Keywords: cognition, PTEN-induced putative kinase1, spatial memory, perirhinal cortex, hippocampus

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder that is characterized by motor deficits such as bradykinesia, postural instability and resting tremor (Bloem et al., 2021; Vazquez-Velez and Zoghbi, 2021). However, many PD patients also experience non-motor symptoms including impairments in cognition and memory (Aarsland et al., 2010, 2017; Goldman et al., 2018; Fang et al., 2020). These impairments appear at or before motor signs in about one third of all PD patients and have a cumulative prevalence of more than 80% overall (Aarsland et al., 2017; Papagno and Trojano, 2018; Fang et al., 2020). Although termed 'mild cognitive impairments' to distinguish these earlier occurring deficits from those associated with Parkinson's disease-related dementia (PDD), these cognitive deficits exact a significant toll on patients' quality and activities of daily life (Leroi et al., 2012; Kudlicka et al., 2014; Oosterveld et al., 2015; Rodriguez-Blazquez et al., 2015; Barone et al., 2017; Saredakis et al., 2019). They also predict a more rapid and more severe clinical course of cognitive and motor decline and are associated with increased risk for freezing, falls and for developing PDD (Pigott et al., 2015; Mack and Marsh, 2017; Cholerton et al., 2018; Goldman et al., 2018). Equally concerning is the lack of available treatments that can effectively combat these impairments (Goldman and Weintraub, 2015; Mack and Marsh, 2017; Goldman et al., 2018; Fang et al., 2020). Using a series of novel object recognition-based paradigms, the studies presented here provide behavioral evidence indicating the potential suitability of rats bearing knockout of the PTEN (phosphatase and tensin homolog)-induced putative kinase1 gene (*Pink1*–/–) for facilitating the preclinical studies that are necessary to better understand and better treat cognitive and memory dysfunction in PD.

Currently, there are few available therapeutic options that are effective in preventing or slowing the progression of cognitive and memory decline in PD (Goldman and Weintraub, 2015; Mack and Marsh, 2017; Goldman et al., 2018; Fang et al., 2020). Thus, in addition to clinical trials, animal and especially rodent models are being used to support controlled investigations into the risk factors and pathophysiological mechanisms that contribute to cognitive disturbance in PD and to test new ways of mitigating them (Fan et al., 2021). There are several reasons to predict that *Pink1*–/– rats may be well-suited for these purposes. First, *Pink1*–/– rats have construct validity for the recessively inherited loss of function *Pink1* mutations that are the second most common mutation among autosomal recessive forms of PD; these mutations are also causally linked to early onset familial cases of PD (Valente et al., 2004; Kumazawa et al., 2008; Scarffe et al., 2014). In addition to disrupting mitochondrial function (Borsche et al., 2020), *Pink1* mutations in PD patients have also been shown to increase central nervous system vulnerability to reactive oxygen species, to dysregulate dopamine (DA) synthesis and reuptake (Gautier et al., 2008; Bus et al., 2020; Goncalves and Morais, 2021), to induce ferritin accumulation and iron toxicity in midbrain DA neurons (Hagenah et al., 2008) and to promote alpha-synuclein aggregation (LSamaranch et al., 2010; Takanashi et al., 2016; Nybo et al., 2020). A rapidly growing literature documents characteristics similar to these in *Pink1* knockout rat lines (Urrutia et al., 2014; Villeneuve et al., 2016; Creed and Goldberg, 2018; Ren and Butterfield, 2021). Further, although the data are not entirely consistent (de Haas et al., 2019), this rat strain has also been shown to undergo progressive loss of midbrain DA and brainstem norepinephrine (NE) neurons (Dave et al., 2014; Grant et al., 2015; Villeneuve et al., 2016; Cullen et al., 2018; Kelm-Nelson et al., 2018b). Earlier occurring, presumed compensatory changes in neostriatal concentrations and/or basal and potassium stimulated release of DA, acetylcholine (ACh), serotonin and other PD-relevant neurotransmitter systems have also been reported (Dave et al., 2014; Creed et al., 2019). Finally, *Pink1*–/– rats display behavioral deficits in motor and non-motor functions that mimic those experienced by PD patients. For example, in addition to age-related decline in gait coordination and grip strength (Dave et al., 2014), *Pink1*–/– rats also demonstrate early-appearing deficits in sensorimotor cranial/otolaryngeal functions that negatively impact vocalizations, chewing and swallowing (Grant et al., 2015; Cullen et al., 2018; Kelm-Nelson et al., 2018a, 2021). In addition, *Pink1*–/– rats also show behavioral correlates reflecting increased anxiety, e.g., changes in distress vocalizations, social approach, open vs. closed arm entries in elevated plus maze testing (Kelm-Nelson et al., 2018a; Cai et al., 2019; Hoffmeister et al., 2021, 2022). This suggests face validity for the mood disturbances that are common in PD patients– including those with causal mutations in the *Pink1* gene (Ephraty et al., 2007; Ricciardi et al., 2014). However, there has been little systematic effort to determine whether *Pink1*–/– rats also model PD-relevant cognitive or memory phenotypes. This is despite evidence that among genetically determined forms of PD, patients with *Pink1* mutations have the greatest incidence of cognitive dysfunction and decline (Piredda et al., 2020; Gonzalez-Latapi et al., 2021). To fill this gap in knowledge, longitudinal testing using Novel Object Recognition (NOR), Novel Object Location (NOL), and Object in Place (OiP) paradigms was used to determine whether and when *Pink1*–/– rats express deficits similar to the impairments in visual recognition memory and//or visuospatial information processing that commonly occur in PD patients (Owen et al., 1993; Higginson et al., 2005; Possin et al., 2008; Fang et al., 2020; Fernandez-Baizan et al., 2020).

Object recognition-based behavioral paradigms are well-validated and widely used for evaluation of mnemonic constructs similar to those that are frequently at risk in neuropsychiatric disorders including Alzheimer's disease, schizophrenia, PD and others (Grayson et al., 2015). Further, these single-trial tasks require no formal training, leverage spontaneous behaviors, are minimally stressful and can require minimal physical exertion (Ennaceur, 2010; Luine, 2015; Aggleton and Nelson, 2020; Chao et al., 2020). These features are especially important for studying cognition and memory in preclinical models of PD where potentially confounding disease-related features of anhedonia, mood disturbance and motor impairment may be present. Finally, there is a rich, task-specific literature for object recognition paradigms describing the brain regions, networks and neurochemical systems that provide essential support for the different forms of recognition memories that these paradigms

measure (Dere et al., 2007; Brown et al., 2012; Aggleton and Nelson, 2020; Barker and Warburton, 2020a,b; Chao et al., 2020). Thus, there is a powerful interpretive framework at hand for gaining insights into the neural circuits that may be most affected by pathophysiology and where targeted therapeutics may be most beneficial. Given these benefits, it is not surprising that NOR, NOL and to a lesser extent OiP tasks continue to be widely used to assess cognitive deficits in a range of rodent models of PD (Grayson et al., 2015; Johnson and Bobrovskaya, 2015; Haghparast et al., 2018; Ikram and Haleem, 2019; Kyser et al., 2019; Bharatiya et al., 2020; Boi et al., 2020; Fan et al., 2021; Kakoty et al., 2021; Pinizzotto et al., 2022). This study extends this utilization for the first time to *Pink1–/–* rats in longitudinal comparative evaluations of object recognition memories in single cohorts of knockout and wildtype (WT) male Long Evans rats. In addition to object exploration and discrimination, the analyses presented below include assessments of motor function and affect that were made in conjunction with object recognition testing. Rats were also further evaluated at the beginning and end of the object recognition testing sequence using elevated plus maze testing, analyses of forelimb and hindlimb grip strength and assessments of foot slips in traversing a tapered balance beam.

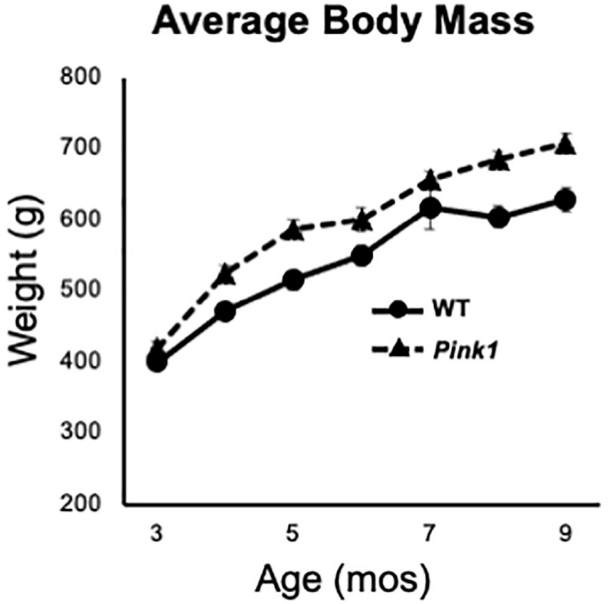


FIGURE 1 | Line graphs showing changes in average weights in grams (g) of the male rats with knockout of the PTEN (phosphatase and tensin homolog)-induced putative kinase1 gene (*Pink1–/–,* triangles, dashed line) and the wild type (WT, circles, solid line) control male rats used in this study as they matured from 3 to 9 months (mos) of age. All rats continued to gain weight as the study progressed. As expected, the average weights of *Pink1–/–* rats were consistently greater than that of the WT cohort.

MATERIALS AND METHODS

Animal Subjects

Male Long Evans rats that were either WT (n = 8) or *Pink1* knockouts [*Pink1–/–*, (LE-Pink1$^{em1Sage-/-}$) n = 16] were purchased at 6 or 7 weeks of age (Envigo, Madison, WI, United States). All rats were double housed by genotype for the duration of the study in standard translucent tub cages (Lab Products, Inc., Seaford, DE, United States) filled with ground corn cob bedding (Bed O' Cobs, The Anderson Inc., Maumee, OH, United States). Rats were kept under a 12-h non-reversed light-dark cycle with food (Purina PMI Lab Diet: ProLab RMH 3000) and water available *ad libitum.* Enrichment objects (Nyla Bones, Nylabone, Neptune, NJ, United States) were also present in each cage. During the intervals when rats were not being behaviorally tested, they spent roughly 1 h per week in groups of 2–6 in a large, dimly lit 6 ft square enclosure that contained tunnels, platforms and other larger scale objects for them to interact with. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Stony Brook University and were performed in accordance with the U.S. Public Health Service Guide for Care and Use of Laboratory Animals to minimize their discomfort. Rats were weighed every month as part of a measure of continued good health and to confirm an expected phenotype of greater body mass in age-matched *Pink1–/–* compared to WT control rats (**Figure 1**).

Behavioral Testing

Habituation and behavioral testing was conducted in a dedicated core facility that includes a central home cage holding room and 5 adjacent 10–12 ft square sound attenuated testing rooms. Each testing room had adjustable high contrast spatial cues on the walls and digital cameras to archive trials. Habituation and testing were conducted during rats' subjective days between the hours of 9:00 am and 1:00 pm under ambient white lighting (∼260 lux).

Apparatus

Object recognition tasks were carried out in open rectangular testing arenas (32 in long, 19 in wide, 13 in high) made of translucent polypropylene. The arenas sat on a table 36 in high. One of the long walls of the arena was made opaque and adjustable, small, high contrast cues were affixed to the outsides of the other three arena walls. These cues as well as distal room cues remained fixed during a given testing period and were rearranged across bimonthly testing sessions.

The elevated plus maze used was constructed of white laminate. It consisted of two open arms (5.5 in × 20.5 in), two closed arms (5.5 in × 20.5 in × 11.25) and an open central platform (5.5 in × 5.5 in). The maze was located 3 feet off the ground.

Grip strength was measured using a San Diego Instruments Animal Grip Strength System outfitted with two push/pull wire mesh force gauges (San Diego Instruments, San Diego, CA, United States).

The tapered balance beam used was composed of black plastic composite (Lafayette Instrument, Lafayette, IN, United States). The top surface was rough to provide grip. The beam was 165 cm in length and tapered in width from 6 to 2 cm. Colored rulers were affixed to the sides of the beam that divided it into wide, medium and narrow thirds. A 2 cm wide ledge ran beside and

below both sides of the beam surface to provide a crutch/step off position for rats to use if needed. Two digital cameras were used to record trials from the left and right sides that rendered all four feet visible.

Habituation

One week after their arrival at Stony Brook University, rats were habituated to handling, to the central room of the testing facility and to gentle transfer between home cages and other enclosures. One week prior to the start of formal behavioral testing, rats were also habituated to the testing arenas and to the opaque start cylinders used in the object recognition tasks. This habituation consisted of daily exposures during which rats were placed in the start cylinder at the center of the arena; after 10 s the cylinder was lifted and rats were given 10 min to explore the empty arena. This was repeated 2–3 times per day at roughly 60 min intervals for 5 days. The first round of object recognition testing began 3 days later; this and all subsequent rounds of object recognition testing began with an initial 5 min habituation trial in the empty arena.

Testing Procedures

Rats were behaviorally tested at 3, 5, 7, and 9 months of age on the NOR, NOL and OiP paradigms; these tasks were given pseudorandom order with 48 h off in between each paradigm. Each trial began by placing rats in an opaque start cylinder located at the center of the arena. After a 10 s delay, the cylinder was lifted and rats were free to explore. Different sets of sample and test objects were used for each task and for each time a given task was delivered. The arena and objects were cleaned with 70% EtOH before and after every trial.

Novel Object Recognition Testing

Novel Object Recognition testing consisted of three 3 min sample trials, each separated by 1 h (**Figure 2A**), and one 3 min test trial separated from the last Sample Trial by 90 min (**Figure 2B**). Rats were returned to home cages during intertrial intervals. During sample trials, two identical objects were placed in adjacent corners of the arena leaving at least 4-inch clearance from the walls. During the test trial rats explored objects that were in the same locations as during sample trials, albeit with one object from the sample trials and one that was novel. The pairs of objects used in NOR testing (**Figures 2C–F**) were similar in overall size and/or shape but differed along dimensions including complexity of shape (e.g., 3 months, **Figure 2C**), composite material (glass or metal, e.g., 5 months, **Figure 2D**), height and color/contrast (e.g., 7, 9 months, **Figures 2E,F**) and/or surface features, e.g., smooth vs. grooved (e.g., 5, 7 months, **Figures 2D,E**). The objects that served as sample vs. novel objects and their locations in the arena were counterbalanced across rats in both groups.

Novel Object Location Testing

Novel Object Location testing consisted of three 3 min sample trials each separated by 1 h (**Figure 3A**) and a 3 min test trial separated from the last sample trial by 1 h (**Figure 3B**). Rats spent

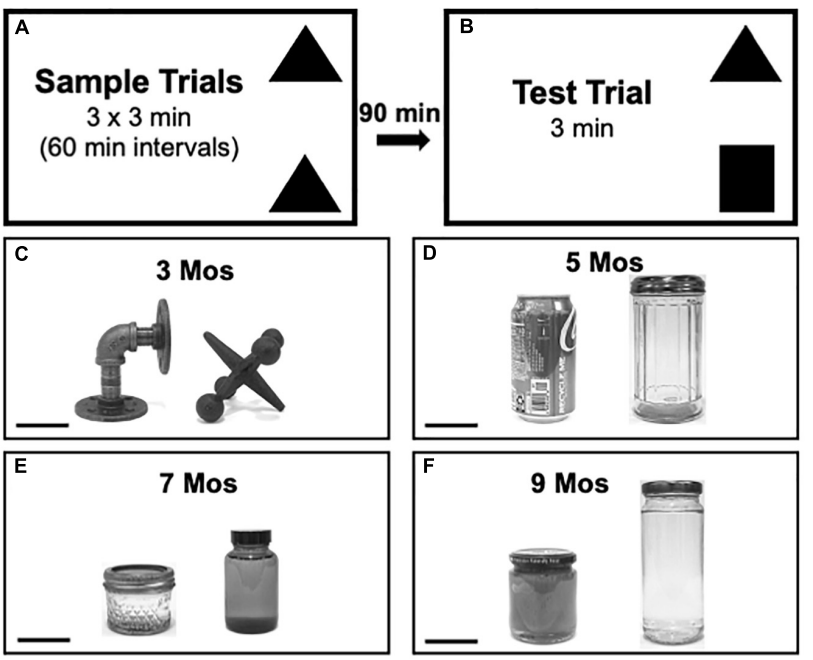


FIGURE 2 | (A,B) Schematic diagrams showing trial structure for the Novel Object Recognition paradigm. **(C–F)** Black and white photographs showing the items that were used as sample or novel objects for testing at each of the four ages evaluated [3, 5, 7, and 9 months (mos) of age]. The objects are displayed at a 45 degree angle relative to each other to provide spatial perspective. The objects used in testing at 3 mos of age **(C)** were made of cast iron. The objects used in testing at 5 mos of age **(D)** were made of aluminum (left) or glass (right). Both items used for testing at 7 **(E)** and 9 mos of age **(F)** were made of glass. Scale bars = 50 mm.

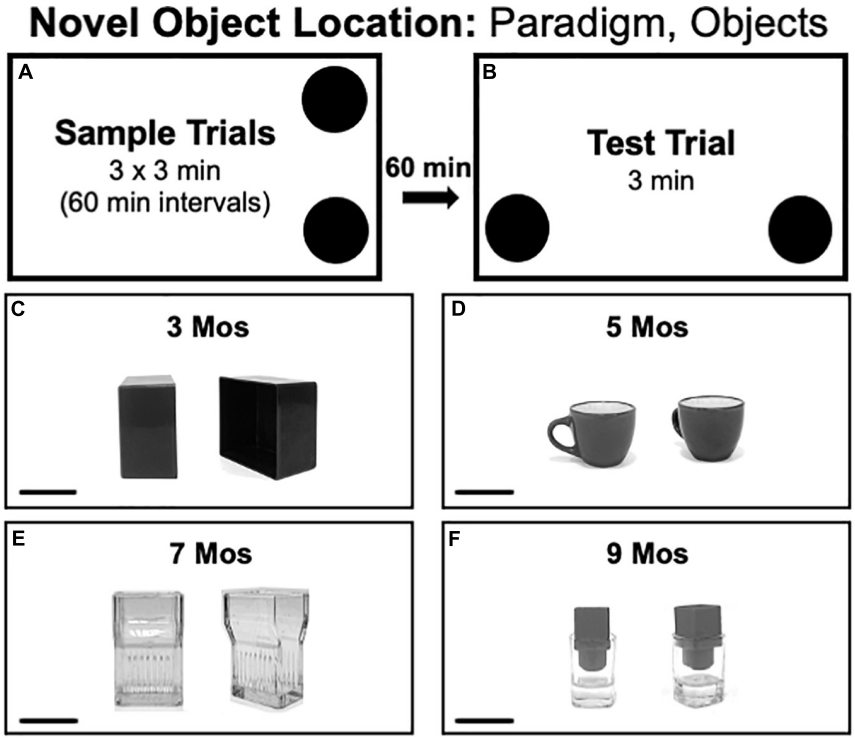


FIGURE 3 | (A,B) Schematic diagrams showing trial structure for the Novel Object Location paradigm. **(C–F)** Black and white photographs show the paired items that were used as sample objects for testing at each of the four ages evaluated [3, 5, 7, and 9 months (mos) of age]. The objects used in testing at 3 mos of age **(C)** were made of plastic. The objects used in testing at 5 mos of age **(D)** were made of ceramic. The objects used in testing at 7 mos of age **(E)** was made of glass micglass, and objects used in testing at 9 mos of age **(F)** were made of glass and plastic. Scale bars = 50 mm.

all intertrial intervals in home cages. During the sample trial, two identical objects were placed in adjacent corners of the arena with a 4-inch clearance from the walls. During test trials rats explored the same two objects but with one located in a corner that was occupied during the sample trial and the other placed in a previously unoccupied corner. Because there is no need to match the valence between objects within trials, the pairs of objects used in NOL had features such as depressions and handles that encouraged close exploration. Across trials, the objects used were made of plastic (**Figure 3C**), ceramic (**Figure 3D**), glass (**Figure 3E**) or a combination of plastic and glass (**Figure 3F**). The arena corners that served as sample vs. novel locations were counterbalanced across rats in both groups.

Object-in-Place Testing

Object-in-Place (OiP) testing consisted of three 3 min sample trials each separated by 5 min (**Figure 4A**) and a 3 min test trial separated from the last sample trial by a 5-min inter-trial interval (**Figure 4B**). Rats were returned to home cages during the intertrial intervals. For sample trials, 4 distinct objects were placed near each of the arena's corners (4-inches from the walls). During Test trials, rats explored the same four objects, albeit with two occupying original positions and two occupying positions that were switched with each other. The groups of objects used in OiP testing were grossly matched in terms of size but each differed from the others along dimensions including color/contrast, composite material (plastic, ceramic, glass or metal), general shape and/or surface features. The objects used for testing at 3, 5, 7, and 9 months of age are shown in **Figures 4C–F**, respectively. The positions and pairs of objects that occupied switched vs. stationary positions were counterbalanced across subjects in both groups.

Elevated Plus Maze Testing

Rats were tested on the elevated plus maze at 3.5 and 9.5 months of age approximately 1 week after completing object recognition testing. At the start of the trial, rats were placed on the center portion of the maze facing away from the handler and were given a single 5 min trial to freely explore. All maze surfaces were cleaned with 70% ethanol before and after each trial.

Grip Strength Testing

Rats were held parallel to the center platform of the apparatus. Once they grasped the forelimb force plate, they were gently pulled backwards, away from it. After they released the forelimb plate, they continued to be drawn across the hindlimb force plate, which rats grabbed onto with hind feet while rats attempted to push forward. Thus, single trials were used to measure forelimb pull strength and hindlimb push/compressive strength. During each session, rats were given three trials that were separated by 30 s to 1 min. The system automatically collects values of maximal force which were used for analyses.

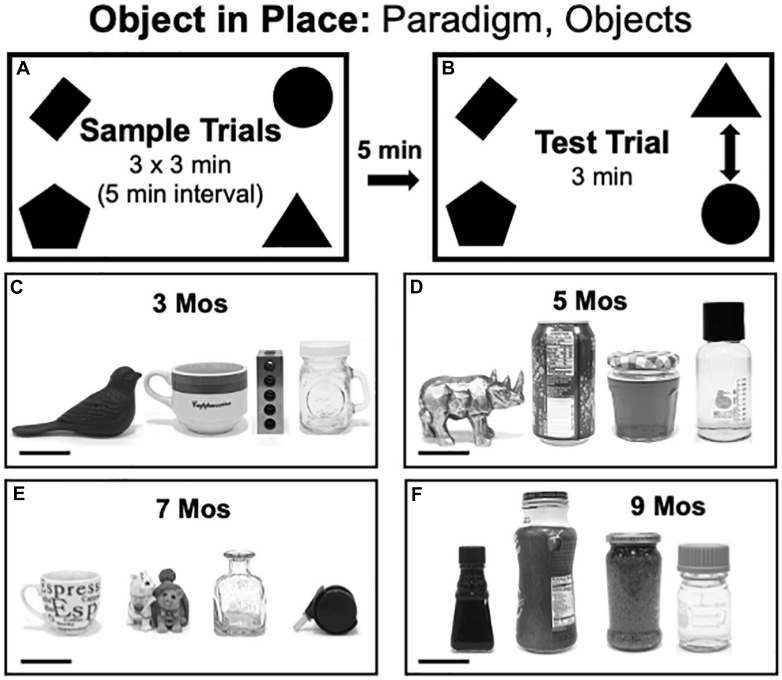


FIGURE 4 | (A,B) Schematic diagrams showing trial structure for the Object in Place paradigm. **(C–F)** Black and white photographs showing the four distinct items used as sample objects for testing at each of the four ages evaluated [3, 5, 7, and 9 months (mos) of age]. From left to right, the objects used in testing at 3 mos of age **(C)** were made of cast iron, ceramic, steel or glass. The objects used in testing at 5 mos of age **(D)** were made of glazed ceramic, aluminum, contoured glass or smooth glass (left to right). The objects used in testing at 7 mos of age **(E)** were made of ceramic, molded plastic, glass, or smooth plastic with metal. The objects used in testing at 9 mos of age **(F)** were all made of glass. Scale bars = 50 mm.

Tapered Balance Beam Testing

Wire tops were removed from rats' home cages and the edge of the open home cage was used to support the narrow end of the balance beam. Rats were removed from the home cage and habituated to beam crossing by first placing them on the narrow end of the beam within a few steps of the home cage. Once they left the beam, they were given 1–2 min in the home cage as reward before being returned to the beam at wider and wider points (farther from the home cage). This was repeated until rats traversed the beam length with minimal stopping. All rats acquired this level of performance quickly, usually in less than three full length runs. Rats were then rested for about 15 min before being given three sequential full length trials.

Data Analysis

Behavioral data were analyzed from digitally recorded trials by trained observers who were blind to genotype/group. Event-capture software [Behavioral Observation Research Interactive Software (BORIS) version 7.8.2, open access] was used to quantify the timing, instances and durations of specific behaviors defined below.

Object Recognition During Sample Trials

Total Exploration

Total time in seconds rats spent actively exploring objects using vibrissae or snout. Sample trial object exploration was additionally evaluated for.

Spatial Bias. Total times in seconds rats spent actively exploring objects located in a given corner, quadrant or half of the arena.

Object Bias. Total times in seconds rats spent actively exploring distinct objects either presented simultaneously (OiP) or counterbalanced across subjects (NOR, NOL).

Object Recognition During Test Trials

Total Exploration

Total time in seconds rats spent actively exploring objects using vibrissae or snout. Test trial object exploration was additionally evaluated for:

Discrimination Index

NOR: Total time (in seconds) rats spent investigating novel (NO) vs. familiar objects (FO), expressed as percent of total object exploration time. This index was calculated by the following formula:

$$[NO] - [FO]/[NO] + [FO]$$

NOL: Total time (in seconds) rats spent investigating objects in new (Nw) vs. original positions (Or), expressed as percent of total object exploration time. This index was calculated by the following formula:

$$[Nw] - [Or]/[Nw] + [Or]$$

OiP: Total time (seconds) rats spent investigating two objects in switched (Sw) compared to original (Or) positions,

expressed as percent of total object exploration time. This index was calculated by the following formula:

[(Sw-Or)/(Sw + Or)].

Other Behaviors

Test trials were analyzed for four major behaviors other than object exploration. The behaviors were defined as below:

- Rearing: total time (seconds) rats spent standing on hind paws either assisted by forepaw contact with objects or walls, or without assistance.
- Grooming: total time (seconds) rats spent preening any part of the head or body.
- Ambulation: total time (seconds) rats made forward motion via steps involving all four paws.
- Stationary: total time (seconds) rats sat at a given location and did not engage in grooming or object investigation.

Elevated Plus Maze

Rats were evaluated for:

- Arm entries: Forward locomotion culminating in all four paws being inside a given arm. Separate counts were made of total arm entries, entries into closed arms and entries into open arms.
- Total times (in seconds) rats spent in open arms, closed arms or on the center platform of the maze.
- Duration (in percent total open arm occupation time) of major activities in open arms.
    - ○ Head dipping- Investigation, with head and shoulders positioned over the edge of the open arm.
    - ○ Ambulation- As per "Other Behaviors" above
- Duration (in percent total closed arm occupation time) of major activities in closed arms.
    - ○ Rearing- As per "Other Behaviors" above
    - ○ Grooming-As per "Other Behaviors" above
    - ○ Ambulation- As per "Other Behaviors" above
    - ○ Stationary-As per "Other Behaviors" above
- Duration (in percent total center platform occupation time) of major activities in center platform.
    - ○ Stretch attend/scanning- total times rats spent making forward and back or side-to-side exploratory movements of the forebody with hindlimbs and tail remaining in place.
    - ○ Rearing- As per "Other Behaviors" above
    - ○ Ambulation- As per "Other Behaviors" above
    - ○ Head dipping- Investigation with head and shoulders positioned over the edge of the open central platform.

Grip Strength

The automated system was used to measure pull force of forelimbs and push/compressive force of hindlimbs. Values of maximal force recorded were normalized to body mass/weight prior to analysis.

Tapered Balance Beam

Rats were evaluated for foot slips made while traversing the full length of the balance beam. Data were collected separately for wide, middle and narrow portions of the beam. Because foot slips were rare for rats in both groups, these data were collapsed into measures of total numbers of foot slips per traversal for analysis. The percentage of rats per group committing some vs. no foot slips was also recorded.

Statistics

Statistical analyses were performed using IBM SPSS, Version 25 (SPSS, Inc., Chicago, IL, United States). The data were first assessed for descriptive statistics, including Levine's *F*-test for equality of variance. Comparisons of single measures across group/genotype were made using one-way analyses of variance (ANOVA), comparisons of measures across age were made using within-groups, one-way ANOVAs with repeated measures designs and comparisons of multiple measures made across groups used two-way repeated measures ANOVA. For all repeated measures comparisons, Mauchly's test for sphericity of the covariance matrix was applied and degrees of freedom were adjusted as indicated using the Huynh-Feldt epsilon. Discrimination index (DI) data were additionally evaluated within groups using one sample *t*-tests to determine whether DI values were significantly different than zero, and relationships between individual measures of DI and rats' total times spent exploring objects during sample and test trials were also assessed within groups by calculating Pearson's correlation coefficients. All comparisons were additionally evaluated for effect sizes by calculating eta squared (η^2) for ANOVAs or using Cohen's *D* for *t*-tests.

RESULTS

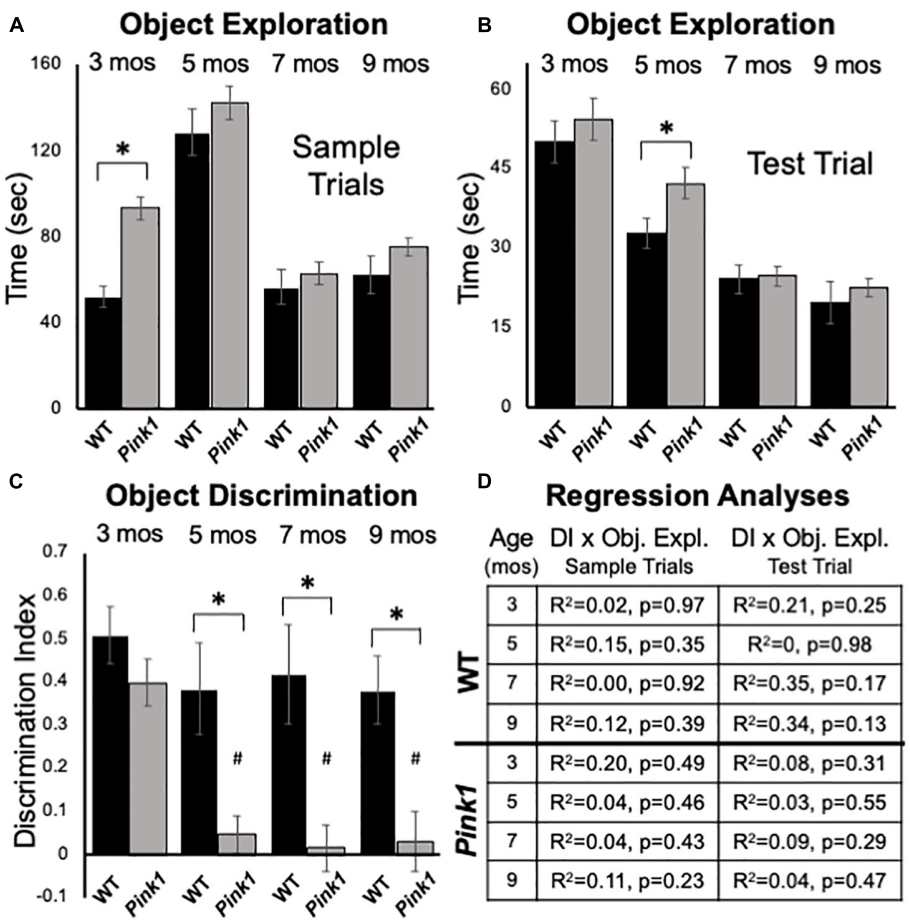
Novel Object Recognition

Sample Trial Object Exploration

At 3 months of age, *Pink1*–/– rats spent nearly twice as much total sample trial time investigating objects as did WT controls (*Pink1*–/– = 93 s, WT = 52 s, **Figure 5A**); this difference was significant [ANOVA, $F_{(1,21)} = 26.61$, $p < 0.001$, $\eta^2 = 0.56$]. During subsequent testing *Pink1*–/– rats also tended to spend more time exploring sample objects (**Figure 5A**). However, the differences in sample observation times seen at these later ages were relatively small (5 months = 142 vs. 128 s; 7 months = 62 vs. 56 s; 9 months = 75 vs. 75 s) and were not significantly different across genotype ($\eta^2 = 0.01$–0.05).

Sample Trial Object Bias

At all ages tested, rats in both groups tended to divide total times investigating sample objects more or less equally among the two objects present. This was confirmed in a series of within-groups repeated measures ANOVAs that in most cases found no significant main effects of object position on object exploration (3 *Pink1*–/–, WT, $\eta^2 = 0.007, 0.013$; 9 months: *Pink1*–/–, WT, $\eta^2 = 0.023, 0.15$). The only exception occurred among WT controls at 5 months of age. For this timepoint, a significant main



	Age (mos)	DI x Obj. Expl. Sample Trials	DI x Obj. Expl. Test Trial
WT	3	R^2=0.02, p=0.97	R^2=0.21, p=0.25
	5	R^2=0.15, p=0.35	R^2=0, p=0.98
	7	R^2=0.00, p=0.92	R^2=0.35, p=0.17
	9	R^2=0.12, p=0.39	R^2=0.34, p=0.13
Pink1	3	R^2=0.20, p=0.49	R^2=0.08, p=0.31
	5	R^2=0.04, p=0.46	R^2=0.03, p=0.55
	7	R^2=0.04, p=0.43	R^2=0.09, p=0.29
	9	R^2=0.11, p=0.23	R^2=0.04, p=0.47

FIGURE 5 | Novel Object Recognition data. Bar graphs showing total times in seconds (sec) that wild type (WT, black bars) and rats with knockout of the PTEN (phosphatase and tensin homolog)-induced putative kinase1 gene (*Pink1–/–*, gray bars) spent actively exploring objects during Sample Trials **(A)** and during Test Trials **(B)** in testing at 3, 5, 7, and 9 months (mos) of age. Overall, *Pink1–/–* rats spent more time than WT rats investigating objects; asterisks identify these differences as significant at 3 mos of age for Sample Trials **(A)**, and for 5 mos of age for Test Trials **(B)**. **(C)** Bar graphs showing calculated discrimination index (DI) for WT (black bars) and *Pink1–/–* rats (gray bars). This measure of object recognition memory was similar in rats of both genotypes at 3 months of age. At all other ages, DIs were significantly greater (*) in WT than in *Pink1–/–* rats. Within-groups comparisons of DI across age showed that in *Pink1–/–* rats, DIs measured at 5, 7, and 9 months of age were significantly lower (#) than DI measured at 3 months of age. **(D)** Tables showing R^2 and *p*-values for regression analyses that compared DIs to total sample object exploration times and to test trial object exploration times in WT and *Pink1–/–* rats at each age tested. No significant or near significant correlations were found among these measures.

effect of Object Position [$F_{(1,2)} = 9.16$, $p = 0.019$, $\eta^2 = 0.57$] was found that was driven by WT rats dividing total sample object investigation times among the two objects present according to a ratio of roughly 60–40. There were no significant group differences noted in sample trial object exploration times based on which object was used as sample at any age ($\eta^2 = 0$–0.064).

Test Trial Object Exploration

Rats of both genotypes spent roughly 10–30% of test trial times exploring objects (**Figure 5B**). On average *Pink1–/–* rats spent more time exploring objects than WT controls (**Figure 5B**). However, these differences were generally less than 5 s (3 months = 54 vs. 50 s; 5 months = 42 vs. 33 s; 7 months = 25 vs. 24 s; 9 months = 22 vs. 20 s). Analyses of variance showed that main effects of genotype on this measure were only significant at 5 months of age [$F_{(1,21)} = 4.44$, $p = 0.047$, $\eta^2 = 0.17$].

Test Trial Object Discrimination

Rats of both genotypes demonstrated robust discrimination of novel compared to familiar objects at 3 months of age (WT DI = 0.51; *Pink1–/–* DI = 0.40, **Figure 5C**). An ANOVA confirmed that there were no significant differences between these two values ($\eta^2 = 0.068$) and one-sided *t*-tests showed that DI values for rats of both genotype were significantly different/greater than zero [WT: $t(7) = 7.56$, *Pink1–/–*: $t(14) = 7.26$, $p < 0.001$, $d = 0.19$ and 0.21, respectively]. During subsequent testing, WT rats maintained robust levels of novel object discrimination (DIs = 0.38 – 0.42, **Figure 5C**).

A within-groups, repeated measures ANOVA further confirmed that DI values in this group were unchanged from 3 to 9 months (η^2 = 0.11), and one-sided t-tests showed that all DI values were significantly different/greater than zero [$t(7)$ = 3.6–4.8, p < 0.001–0.006, d = 0.22–0.31]. In contrast, novel object discrimination in 5-month-old *Pink1*–/– rats dropped dramatically to very low levels that were maintained up to 9 months of age (DIs = 0.02–0.05, **Figure 5C**). A within-groups ANOVA identified significant impacts of age on DI in the *Pink1*–/– group [$F_{(3,42)}$ = 11.12, p < 0.001, η^2 = 0.44] and follow-up comparisons confirmed that the DIs measured at 5, 7, and 9 months in these knockout rats were significantly lower than that measured at 3 months (p < 0.001 for all ages). A series of one-sided t-tests also showed that none of the DI values measured in *Pink1*–/– rats at 5–9 months of age were significantly different than zero (d = 0.17–0.27). Finally, ANOVAs that compared groups identified main effects of genotype on DI in rats that were significant at 5, 7, and 9 but not 3 months of age [5 months: $F_{(1,21)}$ = 12.05, P = 0.002; 7 months: $F_{(1,20)}$ = 13.00, p = 0.002; 9 months: $F_{(1,21)}$ = 9.65, p = 0.005, η^2 = 0.32–0.39, **Figure 5C**]. Regression analyses confirmed that there were no significant or near significant positive correlations between DIs and measures of object exploration during sample or test trials for either genotype at any age (R^2 = 0–0.35, p = 0.13–0.98, **Figure 5D**).

Test Trial: Other Behaviors

Analyses of ambulation, rearing, grooming and remaining stationary during NOR test trials identified significant main effects in the way that animals apportioned test trial times across these behaviors [$F_{(1.52-2.20,\ 30.92-56.60)}$ = 17.43–33.41, p < 0.001 for all, η^2 = 0.45–0.61]. For all testing except at 7 months of age, significant main effects of genotype [$F_{(1,\ 21)}$ = 4.66, p = 0.043, η^2 = 0.18] and/or significant interactions between genotype and behavior [$F_{(1.194-2.69,\ 40.20-56.57)}$ = 3.76–9.64, p < 0.001–0.027, η^2 = 0.15–0.32) were also found. Follow up comparisons further showed that at every testing age *Pink1*–/– rats spent significantly less time grooming than the WT rats (3 months = 1.4 vs. 25 s, p < 0.001; 5 months = 6.3 vs. 25 s, p < 0.001; 9 months = 11 vs. 32 s, p = 0.001). At 9 months of age *Pink1*–/– rats were also found to spend significantly more time ambulating (29 vs. 19 s, p = 0.017) and rearing (57 vs. 29 s, p = 0.002) and significantly less time remaining stationary (60 vs. 81 s, p = 0.011) than WT controls. At all other testing ages, rats of both genotypes spent similar amounts of NOR test trial times engaged in these activities.

Novel Object Location

Sample Trial Object Exploration

At 3 months of age, an ANOVA confirmed that the *Pink1*–/– rats spent significantly more total sample trial times investigating objects than WT subjects [121 vs. 82 s, $F_{(1,21)}$ = 7.05, p = 0.015, η^2 = 0.25, **Figure 6A**]. However, group differences (*Pink1*–/– vs. WT) in sample object exploration at subsequent ages were all negligible (5 months = 54 vs. 55 s; 7 months = 39 vs. 40 s; 9 months = 48 vs. 53 s) and were not significant (η^2 = 0.001–0.013).

Sample Trial Object Bias

Analyses of total sample trial object explorations as functions of object position showed that rats of both genotypes investigated the two sample objects present to similar extents. The largest difference seen in exploring one vs. the other object was for 3-month-old WT rats, where an average difference on the order of about 10 s was seen. However, within-groups repeated measures ANOVAs confirmed that this difference and most others were not significant (η^2 = 0.001–0.22). The single exception was for 9 months old WT rats, where relatively small differences in the amounts of times spent investigating objects located in each the two corners (23 vs. 29 s) proved significant [$F_{(1,7)}$ = 5.96, p = 0.045, η^2 = 0.46].

Test Trial Object Exploration

Analyses of total times spent exploring objects during NOL test trials showed that *Pink1*–/– rats generally spent more time investigating objects than the WT controls (3 months = 38 vs. 14 s; 5 months = 21 vs. 24 s; 7 months = 18 vs. 13 s; 9 months = 19 vs. 13 sec, **Figure 6B**). These group differences were significant for rats at 3 [$F_{(1,21)}$ = 19.58, p < 0.001, η^2 = 0.50] and 9 months of age [$F_{(1,21)}$ = 6.06, p = 0.023, η^2 = 0.22] but did not reach a critical difference at the other two testing ages (η^2 = 0.01, 0.15).

Test Trial Object Discrimination

At 3 months of age, rats of both genotypes showed modest discrimination of objects in novel compared to familiar locations (WT DI = 0.26; *Pink1*–/– DI = 0.22, **Figure 6C**). Thereafter, object location discrimination rose to and remained at considerably higher levels in WT rats for the duration of testing (DI = 0.49–0.64, **Figure 6C**). In contrast, NOL discrimination in the *Pink1*–/– group remained low across all subsequent testing (DI = 0.12–0.17, **Figure 6C**). Nonetheless, one sample t-tests showed that all DI values for both groups were significantly different/higher than zero [WT: $t(7)$ = 2.10- 10.54, p < 0.001–0.04, d = 0.13–0.33; *Pink1*–/–: $t(14)$ = 2.37–3.83, p < 0.001–0.017, d = 0.17–0.25]. Within-groups, repeated measures ANOVAs also showed that there were no significant main effects of age on DIs for rats of either genotype (η^2 = 0.059–0.25). However, across-groups ANOVAs confirmed that DIs in WT rats were significantly higher than those of the *Pink1*–/– cohort at 5, 7, and 9 months of age [$F_{(1,21)}$ = 11.18–21.28, p < 0.001–0.003, η^2 = 0.36–0.50, **Figure 6C**]. Regression analyses also confirmed that for both groups there were no significant or near significant positive correlations between DIs and measures of sample or test trial object exploration at any age (R^2 = 0.001–0.19, p = 0.10–0.91, **Figure 6D**). However, at 9 months of age a significant negative correlation (greater object exploration/lower DI) was identified between total sample trial objective exploration and DI in the Pink1–/– group [$F_{(1,13)}$ = 7.11, p = 0.019, R^2 = 0.35, **Figure 6D**].

Test Trial: Other Behaviors

Analyses of ambulation, rearing, stationary and grooming revealed significant differences in the amounts of time rats of all ages allotted to these activities [$F_{(1.46-2.15,\ 27.81-45.15)}$ = 21.56–43.74, p < 0.001 for all, η^2 = 0.53–0.68]. Significant main effects

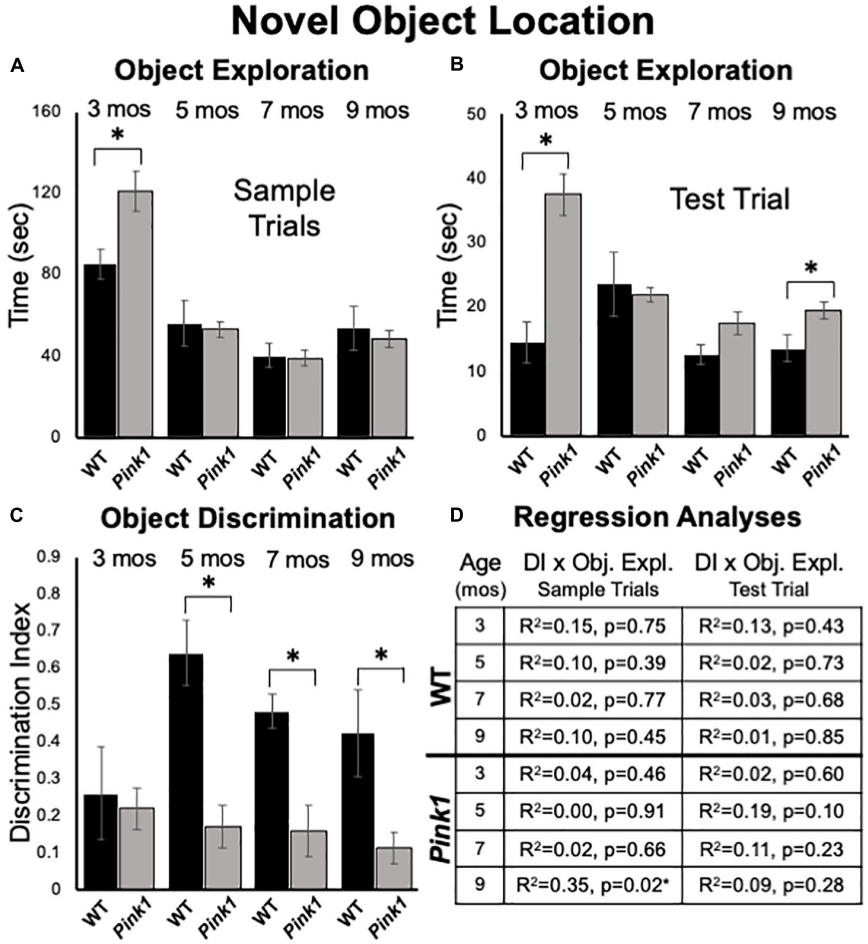


FIGURE 6 | Novel Object Location data. Bar graphs showing total times in seconds (sec) that wild type (WT, black bars) and rats with knockout of the PTEN (phosphatase and tensin homolog)-induced putative kinase1 gene (*Pink1–/–,* gray bars) spent actively exploring objects during Sample Trials **(A)** and during Test Trials **(B)** in testing at 3, 5, 7, and 9 months (mos) of age. In general, *Pink1–/–* rats spent equal or more time than WT rats investigating objects; asterisks identify object exploration times as significantly greater in the *Pink1–/–* compared to WT cohort for testing at 3 mos of age during Sample Trials **(A)**, and for testing at 3 and 9 mos of age during Test Trials **(B)**. **(C)** Bar graphs showing calculated discrimination index (DI) for WT (black bars) and *Pink1–/–* rats (gray bars). This measure of object location memory was similar in rats of both genotypes at 3 months of age. At all other ages, DIs were significantly greater (*) in WT than in *Pink1–/–* rats. **(D)** Tables showing R^2 and *p*-values for regression analyses comparing DIs to total sample object exploration times and to test trial object exploration times in WT and *Pink1–/–* rats at each age tested. No significant or near significant positive correlations were found among these measures. However, a significant negative correlation (*) between increased sample trial object exploration and lower DI values was found for *Pink1–/–* rats at 9 months of age.

of genotype [$F_{(1,20)}$ = 5.40–13.13, p = 0.002-0.031, η^2 = 0.22–0.40] and/or significant interactions between genotype/group and behavior [$F_{(1.55-2.15,\ 30.93-45.15)}$ = 5.67–14.40, $p < 0.001$–0.005, η^2 = 0.21–0.42] were also identified at all testing ages. Although there was some variance in the data, in general, main effects were driven by *Pink1–/–* rats spending more time engaged in active behaviors (rearing, ambulation) and less time being sedentary (stationary, grooming) than WT rats. This was borne out in follow up comparisons that showed *Pink1–/–* rats groomed significantly less than the controls at all ages (3 months = 4 vs. 26 s, $p < 0.001$; 5 months = 5 vs. 17 s, p = 0.002; 7 months = 3 vs. 22 s, p = 0.001; 9 months = 11 vs. 32 s, $p < 0.001$) and spent significantly less time stationary than WT rats in testing at 3 and 5 months of age (3 months = 33 vs. 61 s, $p < 0.001$; 5 months = 32 vs. 64 s, p = 0.004). The *Pink1–/–* group also spent significantly more time ambulating than WT controls in testing at 5 and 7 months of age (5 months = 39 vs. 29 s, p = 0.017; 7 months = 38 vs. 28 s, p = 0.004) and significantly more time rearing at 5 months of age (81 vs. 44 s, p = 0.001).

Object in Place

Sample Trial Object Exploration

At 3 months of age, *Pink1–/–* rats spent significantly more time investigating sample objects than WT rats [127 vs. 91 s, $F_{(1,21)}$ = 11.73, p = 0.003, η^2 = 0.36, **Figure 7A**]. However, at 5 months of age, WT rats spent significantly more time investigating the samples than the *Pink1–/–* cohort [111 vs. 88 s, $F_{(1,\ 21)}$ = 7.66, p = 0.012, η^2 = 0.27, **Figure 7A**]. In testing at 7 and 9 months of age there were no significant main effects of group/genotype on total sample object exploration times between

Pink1–/– and WT rats (7 months = 74 vs. 85 s, η^2 = 0.09; 9 months = 77 vs. 80 s, η^2 = 0.005, **Figure 7A**).

Sample Trial Object Bias

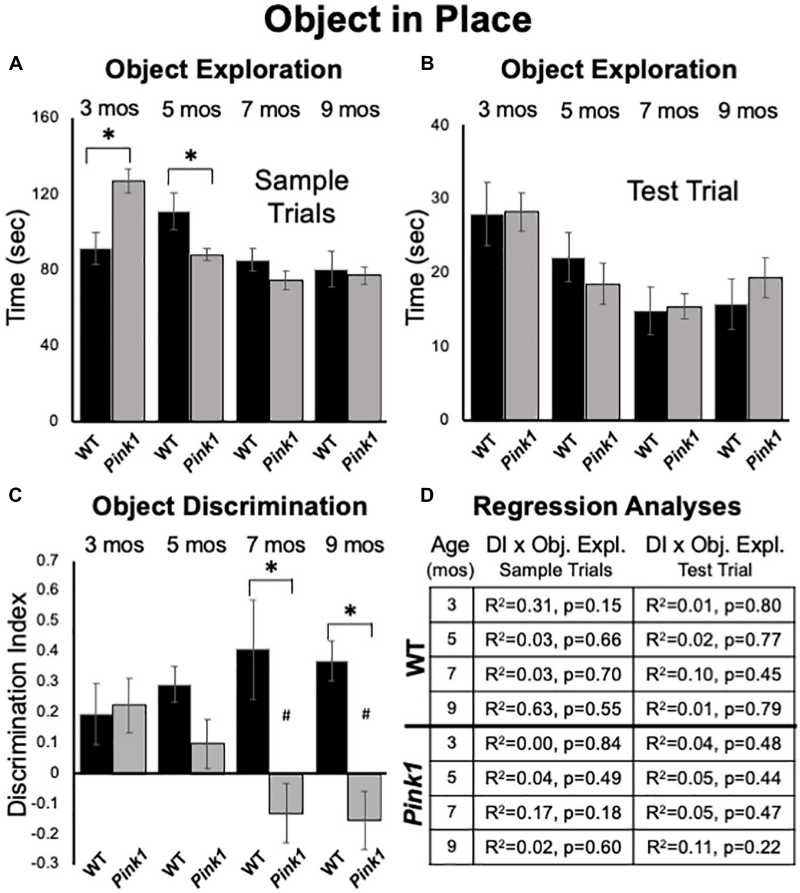
Rats in both groups investigated each of the four sample items present approximately equally and divided observation times similarly across objects located in each of the arena's four corners. Thus, there were no indications of bias based on object type or position. This was confirmed in a series of within-groups, repeated-measures ANOVAs that found no significant main effects of object type or arena corner *(Pink1–/–*: η^2 = 0.011–0.11; Control: η^2 = 0.008–0.24) on measures of object exploration.

Test Trial Object Exploration

Analyses of total times spent exploring objects during test trials showed that *Pink1–/–* and Control rats both spent similar amounts of test trial times exploring objects (3 months = 28 s, both; 5 months = 19 vs. 22 s; 7 months = 15 s, both; 9 months = 19 vs. 16 s, **Figure 7B**). There were no significant main effects of genotype on this measure (η^2 = 0.00–0.031).

Test Trial Object Discrimination

At 3 months of age, WT and *Pink1–/–* rats both showed similar ability to discriminate among objects located in exchanged compared to original positions (WT DI = 0.20; *Pink1–/–* DI = 0.22, **Figure 7C**); one sample *t*-tests showed that all DI's measured in both groups were significantly different/greater than zero [WT: $t(7) = 1.95$, $p = 0.046$ and $d = 0.28$; *Pink1–/–*: $t(13) = 2.52$, $p = 0.013$ and $d = 0.33$, respectively]. However, testing at later time points showed that DIs in WT rats tended to incrementally increase (5 months DI = 0.29; 7 months DI = 0.41; 9 months DI = 0.37, **Figure 7C**). One sample *t*-tests confirmed



	Age (mos)	DI x Obj. Expl. Sample Trials	DI x Obj. Expl. Test Trial
WT	3	R^2=0.31, p=0.15	R^2=0.01, p=0.80
	5	R^2=0.03, p=0.66	R^2=0.02, p=0.77
	7	R^2=0.03, p=0.70	R^2=0.10, p=0.45
	9	R^2=0.63, p=0.55	R^2=0.01, p=0.79
Pink1	3	R^2=0.00, p=0.84	R^2=0.04, p=0.48
	5	R^2=0.04, p=0.49	R^2=0.05, p=0.44
	7	R^2=0.17, p=0.18	R^2=0.05, p=0.47
	9	R^2=0.02, p=0.60	R^2=0.11, p=0.22

FIGURE 7 | Object-in-Place data. Bar graphs showing total times in seconds (sec) that wild type (WT, black bars) and rats with knockout of the PTEN (phosphatase and tensin homolog)-induced putative kinase1 gene (*Pink1–/–*, gray bars) spent actively exploring objects during Sample Trials **(A)** and during Test Trials **(B)** in testing at 3, 5, 7, and 9 months (mos) of age. In general, the amounts of time spent exploring objects were comparable among the *Pink1–/–* WT groups. However, asterisks in **(A)** identify object exploration times that were significantly greater in the *Pink1–/–* compared to WT cohort for testing at 3 mos of age, and that were significantly greater in the WT compared to *Pink1–/–* rats for testing at 5 mos of age during. **(C)** Bar graphs showing calculated discrimination index (DI) for WT (black bars) and *Pink1–/–* rats (gray bars). This measure of integrated object recognition memory was similar in rats of both genotypes at 3 and 5 months of age. At all other ages, DIs were significantly greater (*) in WT than in *Pink1–/–* rats. Within groups comparisons of DI across age showed that in *Pink1–/–* rats, DIs measured at 7 and 9 months of age were significantly lower (#) then DI measured at 3 and 5 months of age. **(D)** Tables showing R^2 and *p*-values for regression analyses comparing DIs to total sample object exploration times and to test trial object exploration times in wild type and *Pink1–/–* rats at each age tested. No significant or near significant correlations were found among these measures.

that all WT DI values were significantly different/greater than zero [$t(7) = 2.49–5.77$, $p < 0.001–0.021$, $d = 0.18–0.46$]. However, within-groups repeated measures ANOVAs showed that the incremental increases in DI observed across age were not significant ($\eta^2 = 0.09$). In contrast, average DIs in the *Pink1*–/– group showed a stepwise decline from 5 to 9 months of age (5 months DI = 0.10; 7 months DI = –0.13; 9 months DI = –0.15, **Figure 7C**). One sample *t*-tests showed that DI's measured across this interval were not significantly different than zero ($d = 0.32–0.37$) and a within-groups repeated measures ANOVAs confirmed DI's significantly declined with age [$F_{(3,30)} = 5.18$, $p < 0.005$, $\eta^2 = 0.34$]. Follow-up comparisons specifically identified DI's measured at 3 and 5 months as significantly greater than those measured at 7 and 9 months of age ($p = 0.003–0.047$, **Figure 7C**). Finally, across-groups ANOVAs showed that while DIs between the WT and *Pink1*–/– rats were initially similar, their diverging trajectories culminated in significant group differences at 7 and 9 months of age [$F_{(1,21)} = 8.94–13.95$, $p < 0.001–0.008$, $\eta^2 = 0.33–0.40$, **Figure 7C**]. Importantly, regression analyses confirmed that there were no significant or near significant positive correlations between DI and measures of object exploration during sample or test trials for any group at any age ($R^2 = 0.004$-0.31, $p = 0.15–0.84$, **Figure 7D**).

Test Trial: Other Behaviors

Analyses of ambulation, rearing, stationary behavior and grooming revealed significant differences in the amounts of time rats of all ages allotted to these activities [$F_{(1.39–3,\ 23.59–60)} = 12.56–90.36$, $p < 0.001$ for all, $\eta^2 = 0.43–0.81$]. However, there were no significant main effects of genotype ($\eta^2 = 0–0.047$) and no significant interactions between genotype/group and behavior ($\eta^2 = 0.03–0.12$) at any testing age.

Elevated Plus Maze

At 3.5 months of age, rats in both groups made comparable numbers of total arm entries (WT = 9.14, *Pink1*–/– = 9.60) that for both genotypes were biased toward entries into closed vs. open arms by more than 2 to 1 (**Figure 8A**). However, a repeated measures ANOVA that compared times spent in a given sector of the maze (open arms, closed arms, center platform, **Figure 8B**) identified significant main effects of maze location [$F_{(2,40)} = 89.12$, $p < 0.001$, $\eta^2 = 0.82$] and a significant interaction between maze location and genotype/group [$F_{(2,40)} = 11.99$, $p < 0.001$, $\eta^2 = 0.38$]. Follow-up pairwise comparisons showed that these effects were driven by WT rats spending significantly more time in closed arms (129 vs. 85 s, $p = 0.005$) and significantly less time in the maze center (136 vs. 190 s, $p < 0.001$) compared to the *Pink1*–/– cohort (**Figure 8B**). Analyses of major behaviors exhibited within each maze location (expressed as percent total times spent within these sectors) also revealed group differences (**Figures 8C–E**). For all three maze compartments, significant main effects were identified for times allotted to particular major behaviors [$F_{(1.61–2.34,\ 32.10–46.69)} = 42.95–51.48$, $p < 0.001$ for all, $\eta^2 = 0.68–0.72$]. However, significant interactions between group and compartment specific behaviors were only significant for open [$F_{(1,20)} = 18.73$, $p < 0.001$, $\eta^2 = 0.48$, **Figure 8C**] and closed [$F_{(2.34,46.69)} = 9.18$, $p < 0.001$, $\eta^2 = 0.32$, **Figure 8D**] arm locations. For the open arms, main effects were driven by WT rats spending about 30% more time head-dipping than *Pink1*–/– rats ($p < 0.001$, **Figure 8C**). For the closed arms, these effects were driven by WT rats spending roughly 12% less time rearing and 16% more time grooming compared to *Pink1*–/– rats ($p < 0.001$ for both, **Figure 8D**). In the center platform, WT and *Pink 1*–/– rats engaged in stretch attend/scanning (41.6, 49.3% of time), ambulating (14.6, 13.4% of time), head dipping (15.7, 9.5% of time) and rearing (10.0, 13.7% of time) similarly (**Figure 8E**). There were no significant group differences in these allotted times ($\eta^2 = 0.065$, 0.001).

At 9.5 months of age, a one-way ANOVA showed that WT rats made significantly fewer total arm entries compared to *Pink1*–/– subjects [5.71 vs. 8.87, $F_{(1,20)} = 7.90$, $p = 0.011$, $\eta^2 = 0.52$, **Figure 8A**]. A repeated measures ANOVA further identified significant main effects of arm type [$F_{(1,20)} = 145.21$, $p < 0.001$, $\eta^2 = 0.88$), a significant main effect of group [$F_{(1,20)} = 8.70$, $p = 0.008$, $\eta^2 = 0.30$] and a significant interaction between these two [$F_{(1,20)} = 5.95$, $p = 0.024$, $\eta^2 = 0.23$]. These effects were driven by *Pink1*–/– rats entering closed arms nearly twice as often (7 vs. 5) as WT rats ($p = 0.002$, **Figure 8A**). In terms of times spent, a repeated measures ANOVA also revealed significant main effects of maze location [open arm, closed arm, center platform, $F_{(1.31,26.20)} = 109.26$, $p < 0.001$, $\eta^2 = 0.85$] and a significant interaction between maze location and group [$F_{(1.31,\ 26.20)} = 6.01$, $p = 0.015$, $\eta^2 = 0.23$). Follow-up pairwise comparisons showed that these effects were driven by WT rats spending significantly more time in closed arms (146 vs. 109 s, $p = 0.023$) and significantly less time in the maze center (141 vs. 178 s, $p = 0.016$) compared to the *Pink1*–/– group (**Figure 8B**). Rats of both genotypes spent roughly 12 s in the open arms of the arena (**Figure 8B**). Finally, analyses of major behaviors exhibited in each portion of the maze found no significant main effects of behavior for open arms ($\eta^2 = 0.10$). For the closed arms and center platform, significant main effects of behavior [$F_{(2.35–3.08,\ 47.01–61.53)} = 12.61–217.62$, $p < 0.001$ for both, $\eta^2 = 0.39–0.92$] and significant interactions between behavior and group [$F_{(2.35–3.08,47.01–61.53)} = 5.93–6.16$, $p < 0.001–0.003$, $\eta^2 = 0.23–0.24$] were found. For the closed arms (**Figure 8D**), these effects were driven by WT rats spending roughly 9% less time ambulating ($p = 0.008$) and about 20% more time grooming compared to the *Pink1*–/– cohort ($p < 0.001$). For the center platform (**Figure 8E**), effects were driven by WT rats spending approximately 10% less time engaged in stretch attend/scanning ($p = 0.008$), about 5% less time rearing ($p = 0.023$) and 5–6% more time head dipping ($p = 0.042$) and ambulating ($p < 0.001$) compared to the *Pink1*–/– group.

Grip Strength

Forelimb and hindlimb grip strength was measured in rats at 3.5 and 9.5 months of age. All measurements were normalized to total body weight. At both timepoints,

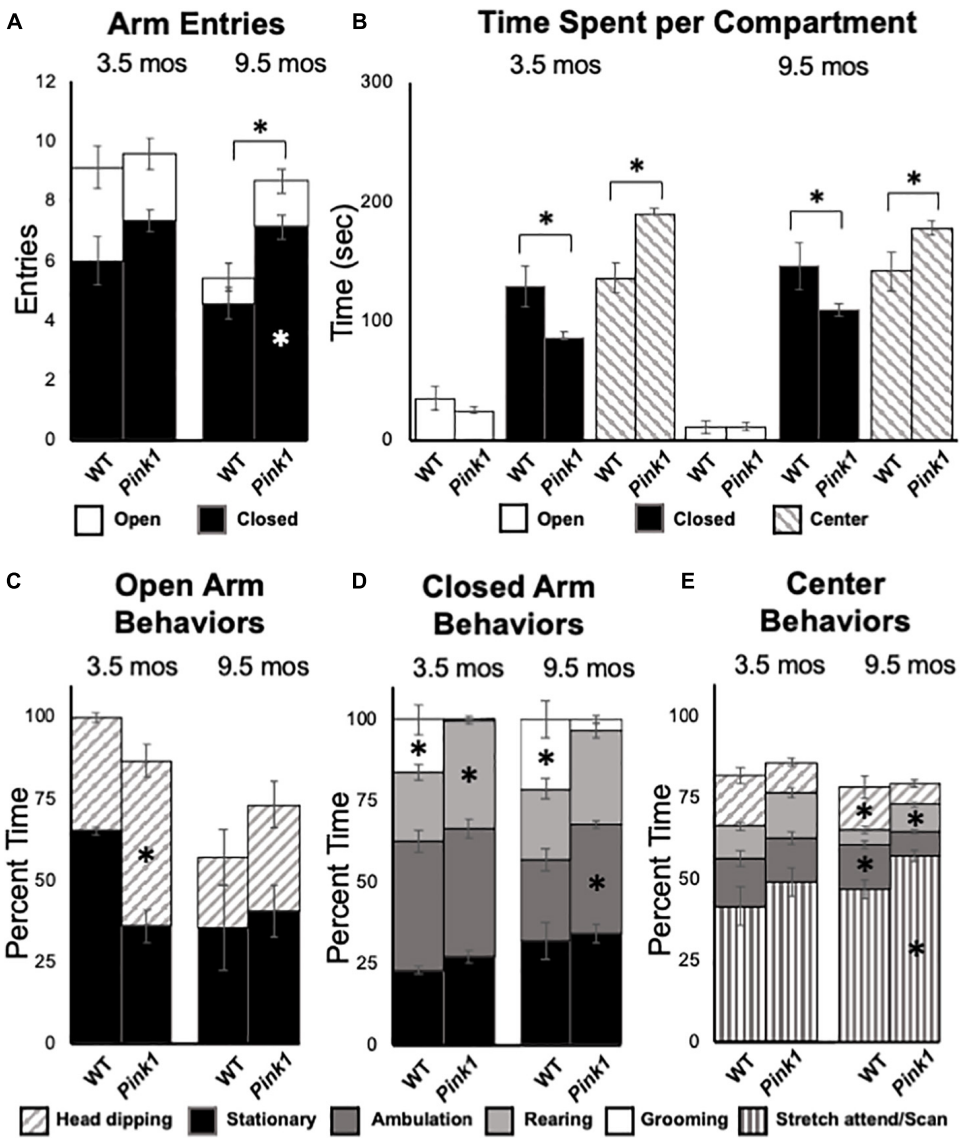


FIGURE 8 | Elevated Plus Maze data. **(A)** Stacked bar graphs showing total arm entries divided into total entries made into open (white) and closed (black) arms for wild type (WT) and rats with knockout of the PTEN (phosphatase and tensin homolog)-induced putative kinase1 gene (*Pink1–/–*) for testing at 3.5 and 9.5 months (mos) of age. The black asterisk shows that at 9.5 mos of age, rats in the *Pink1–/–* group made significantly more closed arm entries than WT rats; the white asterisk shows that *Pink1–/–* rats also made significantly more entries into closed arms than WT rats. **(B)** Bar graphs showing total amounts of time WT and *Pink1–/–* rats spent in the open arms (white), closed arms (black) and the center platform (striped) of the maze during testing at 3.5 and 9.5 mos of age. Asterisks show that at both 3.5 and 9.5 months of age, *Pink1–/–* rats spent significantly less time in closed arms and significantly more time on the center platform than WT rats. Stacked bar graphs showing percentages of total times *Pink1–/–* and WT rats spent on major behaviors within the open arms **(C)**, closed arms **(D),** and center platform **(E)** during testing at 3.5 and 9.5 mos of age. Major behaviors examined included stationary behavior (black), ambulation (dark gray), rearing (light gray), grooming (white) head dipping (slanted stripes) and engaging in stretch-attend/scanning (vertical stripe) Significant group differences in the percentages of total times that rats devoted to a given behavior are marked by asterisks within the bar graphs of the group where significantly more time was spent.

the pull force exerted by forelimbs was greater than pushing/compressive force measured for hindlimbs in both rat groups (**Figures 9A,B**). However, there were no significant main effects of genotype/group on either of these measures at either age tested (forelimb:η^2 = 0–0.11; hindlimb:η^2 = 0–0.03). There were, however, significant main effects of age on normalized grip strength measures for both groups [$F_{(1,23)}$ = 8.21–41.60, p < 0.001–0.009, η^2 = 0.26–0.60]. These main effects were driven by increased normalized hindlimb grip strength forces in 9.5 compared to 3.5-month-old

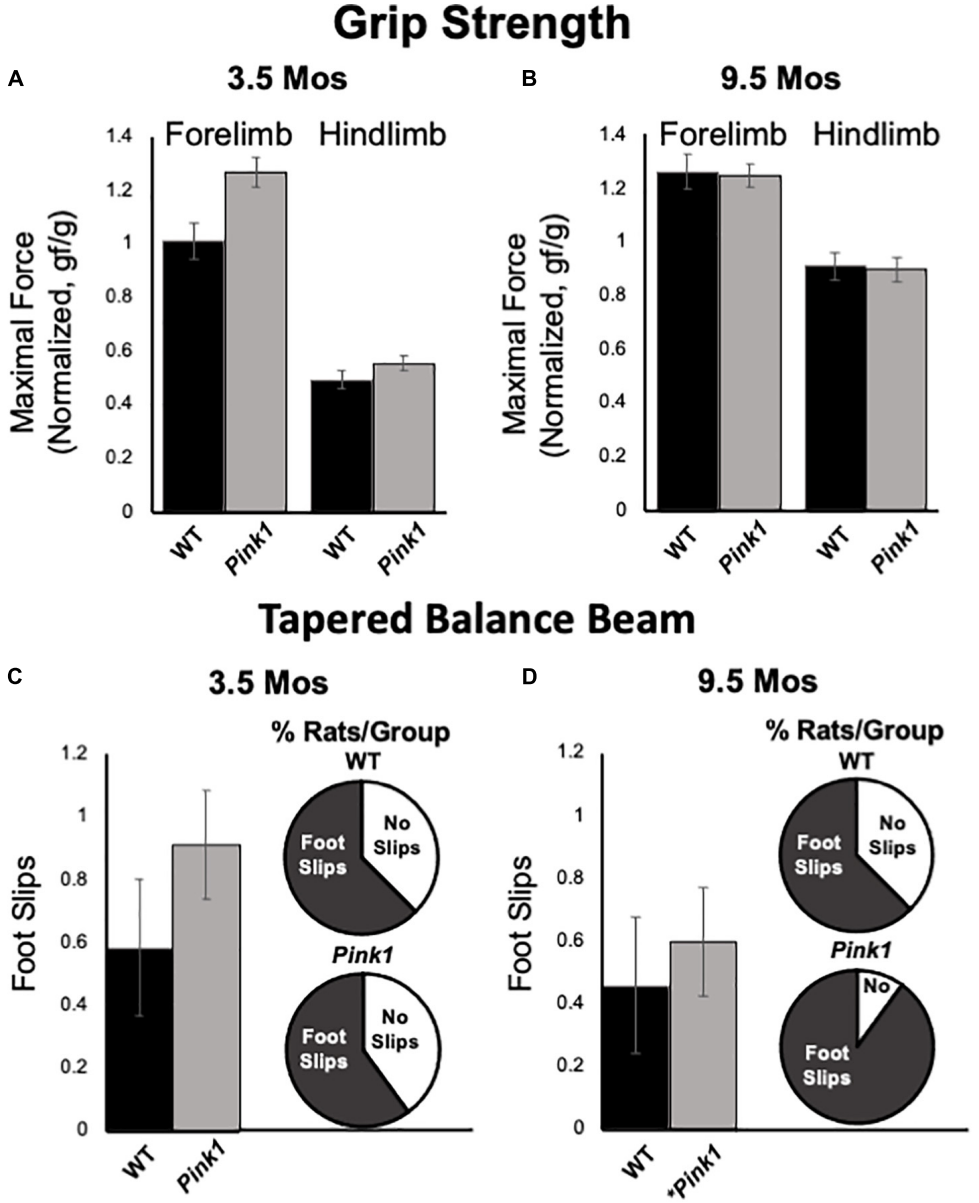


FIGURE 9 | Grip Strength and Tapered Balance Beam data. Bar graphs showing measures of maximum forelimb **(A)** and hindlimb **(B)** grip strength/force normalized to body mass [grams of force (gf)/body weight in grams (g)] in wild type (WT, black bars) and rats with knockout of the PTEN (phosphatase and tensin homolog)-induced putative kinase1 gene (*Pink1–/–*, gray bars) measured at 3.5 and 9.5 months (mos) of age. There were no significant group differences in these measures of muscle strength at either age. Bar graphs showing average total numbers of foot slips committed by WT (black bars) or *Pink1–/–* rats (gray bars) in tapered balance beam traversal trials at 3.5 **(C)** and 9.5 **(D)** mos of age. Pie graph inserts show the proportion/percent of rats in each group that did or did not commit foot slips during the trial. Numbers of foot slips were minimal in both groups at both ages. However, proportionally more *Pink1–/–* rats made foot slips compared to WT rats at 9.5 mos of age. The asterisk on the *X*-axis **(D)** denotes that five rats in the *Pink1–/–* group were removed from testing at 9.5 mos of age due to inability to cross the beam.

rats of both genotypes and increased forelimb grip strength for only WT controls.

Tapered Balance Beam

Tapered balance beam performance was assessed in rats at 3.5 (**Figure 9C**) and 9.5 months of age (**Figure 9D**). It is important to note that five of the *Pink1–/–* rats were no longer able to navigate the beam at the later time point and did not contribute to group data for this age. None of the WT rats were removed from these analyses. Average numbers of foot slips were slightly greater among *Pink1–/–* that did complete the task compared to WT rats at both ages. These differences, however, were small, not significant (η^2 = 0.01–0.02) and represented small numbers of actual step offs/slips. The numbers/percentages of animals in

each group that did and did not commit step offs/foot slips were also assessed; these percentages were similar among both groups of rats at 3.5 months of age (**Figure 9C**) but were greater in the *Pink1*–/– compared to WT rats at 9.5 months of age (**Figure 9D**).

DISCUSSION

Cognitive impairments associated with PD are disabling for a considerable proportion of patients (Leroi et al., 2012; Kudlicka et al., 2014; Rodriguez-Blazquez et al., 2015; Barone et al., 2017). These impairments are also resistant to most available treatments (Goldman and Weintraub, 2015; Mack and Marsh, 2017; Goldman et al., 2018; Fang et al., 2020). Left unchecked, what may initially be mild deficits often progressively worsen and increase the likelihood of patients experiencing freezing and falls and developing PDD– which are leading causes of hospitalization, institutionalization and death among PD patients (Pigott et al., 2015; Peterson et al., 2016; Cholerton et al., 2018). While *Pink1*–/– rats have been shown to recapitulate several key bio-behavioral aspects of PD, it has been largely unknown whether these rats also model the cognitive and/or memory sequelae associated with this disease. To address this question, the present study used three object recognition memory tasks to explore the face validity of this genetic rat model for the deficits in visual recognition memory and/or visuospatial information processing that commonly occur in PD patients (Owen et al., 1993; Higginson et al., 2005; Possin et al., 2008; Fang et al., 2020; Fernandez-Baizan et al., 2020). These analyses revealed significant impairments in the *Pink1*–/– cohort in NOR, NOL, and OiP performance as well as task-specific differences in the progression of these object discrimination/memory deficits across age. To summarize, at 3 months of age, rats of both genotypes showed robust ability to discriminate novel objects. The WT rats sustained these high levels of discrimination across all subsequent testing ages. However, in the *Pink1*–/– cohort, NOR performance declined sharply by 5 months of age and remained extremely low from this age on. In contrast, for the NOL task, rats of both genotypes initially (3 months of age) showed only modest ability to discriminate objects based on their location. However, by 5 months of age, NOL performance in WT rats rose to and remained at higher, more expected degrees discrimination while performance in the *Pink1*–/– group remained moderate to low at all ages. Finally, at 3 months of age the integrative recognition memory functions tapped in the OiP task were moderate in both WT and *Pink1*–/– rats. With successive testing, however, performance in WT rats incrementally increased and performance in *Pink1*–/– rats steadily declined. From these data it is tempting to speculate that knockout of the *Pink1* gene negatively impacts the brain circuits and/or neurochemical systems that are essential for performance in these tasks. However, given previous evidence for motor and affective disturbances in *Pink1*–/– rats (below) it is important to determine whether and how such non-mnemonic factors may have influenced the behavioral outcome measures observed. As discussed below, this was done by incorporating measures of motor function and affect/anxiety into analyses of object recognition task performance, and by bracketing the longitudinal object recognition testing sequence with elevated plus maze testing, measurements of hind- and forelimb grip strengths and assessments of motor coordination in traversing a tapered balance beam.

Object Recognition in *Pink1*–/– Rats: Potential Confounds

As for many preclinical models of PD, it is important that analyses of object recognition memory testing take into consideration the possibility that motor and/or non-motor deficits could be confounding to data interpretation. For example, some muscular/motor effort is required for rats to get to and interact with the objects presented. In addition, disturbances in affect or anxiety can influence animals' willingness to approach or explore objects, particularly those that are unfamiliar (Ennaceur et al., 2005, 2006, 2009). The previous studies showing that *Pink1*–/– rats experience progressive motor deficits and/or show an affective phenotype discussed below underscore the need for careful assessments to assure that the data from object recognition testing reported here reflect cognitive and/or mnemonic status. Accordingly, the present studies incorporated concurrent analyses of motor activity and affect into assessments of object recognition task performance, further evaluated motor function using a grip strength gauge and a tapered balance beam and further evaluated affect and anxiety using elevated plus maze testing.

Motor Function

Pink1–/– rats are notable in part for progressive motor phenotypes. For example, this strain holds important and perhaps unique translational value for recapitulating early cranial and otolaryngeal sensorimotor deficits of PD (Kelm-Nelson et al., 2021). As in PD patients (Ho et al., 1998; Miller et al., 2006a,b), *Pink1*–/– rats have been shown to have difficulty in sustained chewing and swallowing and show diminished vocalizing and vocalization volumes (Grant et al., 2015; Cullen et al., 2018; Kelm-Nelson et al., 2018a; Johnson et al., 2020). Other studies have shown that *Pink1*–/– rats also experience progressive somatic motor deficits. The most potentially concerning for the present studies are data identifying decreased novel open field locomotion and rearing, reduced hindlimb grip strength and increased commission of foot slips in traversing a tapered balance beam that in some (but not all) studies have been seen in *Pink1*–/– rats as young as 4 months of age (Dave et al., 2014; Grant et al., 2015). In the present study, all rats were qualitatively evaluated for ability to freely locomote within the empty testing arena during the habituation/re-habituation trials that preceded every object recognition testing block. Although several *Pink1*–/– rats developed what appeared to be an uncoordinated gait at around 7-month-old, all were able to navigate the relatively small testing arena used and none were excluded from object recognition testing on this basis. Additional motor assessments made in conjunction with object recognition testing also showed that during test trials *Pink1*–/– rats often spent more time rearing and/or ambulating than WT rats. There were also no

significant 'before or after' group differences in measures of fore- or hindlimb grip limb strength or commissions of foot slips on a tapered balance beam showed in *Pink1*–/– rats. It was noted, however, that *Pink1*–/– rats made slightly more foot slips than WT rats, that proportionally more *Pink1*–/– rats committed step offs than WT rats and that 5 of the *Pink1*–/– rats and none of the WT controls had to be removed from tapered balance beam testing at 9.5 months of age due to difficulty in remaining on the widest portions of the beam. Thus, we did find evidence of an emergent motor phenotype in the *Pink1*–/– cohort. However, further, more nuanced analyses are needed to resolve its nature. In the meantime, the qualitative and quantitative data in hand argue against motor impacts in the *Pink1*–/– group as interfering with object exploration or object recognition testing. Importantly, the data also suggest that somatic motor deficits in *Pink1*–/– rats manifest later than do impairments in the cognitive and memory processes tapped in the NOR, NOL, and OiP tasks. This could signal an additional dimension of face validity for the *Pink1*–/– rat model, as impairments in cognition and memory typically present during prodromal phases of illness, i.e., before the onset of measurable motor deficits, in PD patients (Caviness et al., 2007; Pigott et al., 2015; Aarsland et al., 2017; Baiano et al., 2020; Fang et al., 2020).

Anxiety/Affect

Previous observations in *Pink1*–/– rats include behavioral measures suggesting increased anxiety (Kelm-Nelson et al., 2018b; Cai et al., 2019; Hoffmeister et al., 2022). Such traits are potentially relevant for modeling aspects of mood disturbance that are common in PD—including clinical cases that are causally linked to loss of function *Pink1* gene mutations (Ephraty et al., 2007; Ricciardi et al., 2014). However, these traits could also adversely influence performance in object recognition testing. Specifically, while object recognition paradigms themselves are noted for provoking minimal stress or anxiety, baseline differences in anxiety can express as neophobia which reduces rats' contact with objects–especially unfamiliar ones, and significantly erodes the discrimination indices typically used to quantify recognition memories (Ennaceur et al., 2006; Ennaceur, 2010). Among the 'other behaviors' measured during object recognition testing were stationary behavior and grooming. The stationary behaviors observed were distinct from freezing. Accordingly, the significantly reduced times that *Pink1*–/– compared to WT rats spent stationary may be most likely to reflect diminished adaptation or habituation to the testing environment. The grooming that was observed occurred intermittently and included both cephalic and sequential grooming from head to body. Thus, interpretations with respect to decreased grooming in the *Pink1*–/– group leave it uncertain as to whether this difference reflects decreased or increased anxiety. To gain further clarity into this, rats were also tested on an elevated plus maze. Previous studies examining rats at 4 and 12 months of age showed that *Pink1*–/– rats entered and spent significantly more time in closed arms than controls (Hoffmeister et al., 2021). However, in the present study, *Pink1*–/– rats made more entries but spent less time in the closed arms than did WT rats. Further, while neither group spent much time in the open arms, *Pink1*–/– rats spent significantly more time in the center platform than WT rats. Finally, *Pink1*–/– rats spent significantly more time rearing and/or ambulating and less time grooming in the closed arms, and significantly more time engaged in rearing and stretch-attend/scanning and less time head dipping and ambulating in the center platform. Thus, the data are mixed with respect to behaviors classically aligned with increased or decreased anxiety. While these findings provide no indication of a *Pink1*–/– phenotype that would be likely to compromise object recognition testing, there is no question that there are significant differences in the ways in which *Pink1*–/– rats govern behaviors during object recognition and elevated plus maze testing compared to WT rats. Characterizing these differences more thoroughly and resolving their bases are important areas for future investigation.

Impacts of Object Exploration in Time-Limited Trials

The *Pink1*–/– rats assessed in this study were generated on a Long Evans background. Previous studies in this rat strain have demonstrated powerful effects of intermittent sample trial object exposure on subsequent discrimination of novelty. Specifically, it was shown that multiple, shorter exposures to sample objects greatly enhanced rats' sensitivity to novelty demonstrated in test trials compared to a single, longer exposure period (Anderson et al., 2008; Shimoda et al., 2021). These findings drove the decision to incorporate multiple sample trials (3) in the testing protocols used here. Importantly, however, all trials were time-limited and thus subject to unintended impacts of differences in the time spent gaining familiarity with sample objects on later measures of memory strength or recall. Accordingly, analyses included evaluations of any group differences in total times rats spent with objects during both sample and test trial periods. These analyses showed that the generally more active state noted above in *Pink1*–/– compared to WT rats included knockout rats typically spending more to significantly more time actively exploring objects in all trial types. This argues against neophobia and argues against differential exposure to samples as negatively impacting measures of DI in the gene knockout group. The latter was further supported in findings of no significant or near significant positive correlations between the durations of sample or test trial object explorations and DI for any group for any task at any age. Careful analyses of sample trial object explorations also ruled out contributions of innate spatial bias or bias toward object type(s) as contributing to the group, task and age-specific patterns of differential object exploration/discrimination seen in test trials. Rather, as discussed further below, the data in hand may be explained by deleterious consequences of knockout of the *Pink1* gene for the brain circuits and neurochemical systems that mediate object recognition memory functions.

Comparison to Previous Studies

To our knowledge, there has been only one previous assessment of cognition or memory in *Pink1*–/– rats. This study included Barnes maze and NOR testing as part of a larger *in vivo* brain imaging study that examined male rats at 6–8 months of

age (Cai et al., 2019). The data presented were in some cases limited. For example, because the data from Barnes maze testing were collapsed across trials, information about spatial working memory or spatial learning strategies was not available. However, measures of average daily latency to find the goal location showed no differences in performance within or across groups over four sequential testing days. Thus, rats of both genotypes appeared to learn and retain task information similarly. The latter is consistent with findings from other rodent models of PD that often do not recapitulate the long-term reference memory deficits that are characteristic of later stages of disease and PDD (Miyoshi et al., 2002; Da Cunha et al., 2006; Betancourt et al., 2016). For NOR testing, a single sample exposure (5 min) and a 60 min intertrial interval was used. While the *Pink1*–/– group showed no discrimination deficits, these data are difficult to interpret because–perhaps owing to the use of single sample trials, the control cohort showed no preference for novelty. Key methodological details were also lacking, including a description of habituation, information as to whether rats were tested during subjective days or nights, how object exploration was defined and measured and whether rats were tested before or after undergoing *in vivo* imaging. Thus, it is uncertain what may have driven the substantial differences between this prior and the present study where robust deficits in all object recognition memory domains assessed were present in *Pink1*–/– rats by 6–8 months of age.

The present studies used a longitudinal testing strategy to gain insights into the potentially progressive impacts of a PD-relevant gene perturbation on cognition and/or memory. This revealed diverging trajectories in object recognition memory testing performance in WT and *Pink1*–/– rats between 3 and 9 months of age. This was related in part to some unexpected evolutions in object recognition performance in WT rats across this span. Specifically, for NOL testing, WT rats initially showed moderate levels of discrimination that jumped to much higher, asymptotic levels by 5 months of age. Similarly, for OiP, an initially moderate level of discrimination seen in testing at 3 months of age increased, albeit more incrementally, over the next 6 months. While developmental trajectories in object recognition memory performance have been noted, these are described for much younger rats and suggest that adult levels of performance are in place within the first months of life (Reger et al., 2009; Ainge and Langston, 2012; Westbrook et al., 2014; Contreras et al., 2019). Thus, the bases for the age-to-age differences noted in the WT rats of study are unclear. Importantly, however, the generally upward trajectory of their performances indicates that WT rats continued to engage in these tasks and were not negative affected by test–retest contingencies.

Potential Substrates of Object Recognition Impairment in *Pink1*–/– Rats

Longitudinal testing showed that the *Pink1*–/– cohort examined developed robust discrimination deficits in NOR, NOL, and OiP tasks according to task-specific timelines. These rats continue to be tested for motor function. Thus, direct pathophysiological correlates to these behavioral profiles are not available. However, previous multimodal *in vivo* magnetic resonance imaging (MRI) studies in *Pink1*–/– rats have identified significant changes in brain regions and circuits known to be critical for object recognition memories. For example, volumetric analyses have shown that areas including perirhinal and entorhinal cortex, dentate, subicular, CA1 and CA3 fields of the hippocampal formation, nucleus reuniens of the thalamus and several amygdaloid nuclei are significantly smaller in *Pink1*–/– compared to WT rats (Cai et al., 2019). Diffusion weighted MRI has also identified significantly decreased anisotropy in many of these same regions and resting state functional MRI has identified significantly reduced connectivity between neostriatum, midbrain DA regions, hypothalamus and thalamus and increased connectivity between ventral midbrain DA regions and hippocampus in *Pink1*–/– compared to wild type rats (Ferris et al., 2018; Cai et al., 2019). Together these findings show that many of the brain regions and networks known to be critical for object recognition memory (Aggleton and Nelson, 2020; Barker and Warburton, 2020a,b; Chao et al., 2020) are vulnerable to the *Pink1*–/– genotype. In addition, although findings with respect to DA cell body loss have been variable (de Haas et al., 2019), NE cell loss, increased neostriatal concentrations of DA and decreased levels of basal and potassium-stimulated neostriatal release of DA, ACh and others have also been identified in *Pink1*–/– compared to control rats between the ages of 4 and 12 months (Dave et al., 2014; Grant et al., 2015; Villeneuve et al., 2016; Cullen et al., 2018; Creed et al., 2019). Although little is currently known about the status of neurochemistry in other subcortical or cortical regions, these data nonetheless show patterns of dysregulation induced by the *Pink1*–/– genotype that involve neurotransmitters known to play pivotal roles in object recognition memories (Dere et al., 2007; Bus et al., 2020). Further, all of the indices of pathophysiology described above are present in *Pink1*–/– rats over time frames when the results of this study predict that significant impairments in multiple object recognition memory domains would be present. Future studies that combine *in vivo* imaging with behavioral analyses may be in an especially powerful position to map the progression of brain pathophysiology to the evolution of domain specific object recognition memory deficits. Although MRI analyses can be brain wide, current understanding of the points of overlap and divergence among the neural systems that underlie performance in discrete object recognition memory tasks can be used to generate and/or prioritize narrower, more specific hypotheses to be tested by these means.

SUMMARY AND CONCLUSION

Novel object recognition, NOL and OiP testing continues to be extensively used to evaluate recognition memory and visuospatial information processing deficits that are similar to those experienced by PD patients in a range of different preclinical rodent models of disease (Grayson et al., 2015; Haghparast et al., 2018; Kyser et al., 2019; Bharatiya et al., 2020; Boi et al., 2020; Kakoty et al., 2021). The present studies identified robust deficits in all three of these tasks in *Pink1*–/– rats. This is the first demonstration of face validity in this model

for commonly occurring cognitive and memory impairments associated with PD. The longitudinal testing scheme used along with companion assessments of motor and affective function also showed that object recognition memory deficits in *Pink1*–/– rats progressively worsen and precede the onset of potentially confounding motor signs. The need for treatments that prevent or slow the course of cognitive or memory decline in PD– and especially those that do so without interfering with treatment of motor signs, is urgent (Goldman and Weintraub, 2015; Goldman et al., 2018). The present findings of progressive cognitive and memory deficits along with the emergence of motor signs identify *Pink1*–/– rats as well suited for accelerating the pace discovery needed to fill this therapeutic gap. Key directions for future investigations using this model include assessments of long-term object recognition user longer, e.g., 24 h delay periods, evaluation of additional at-risk behavioral domains including executive function and exploration of potential face validity of *Pink1*–/– rats for the sex differences that characterize the incidence and severity of mild cognitive impairments in PD (Janvin et al., 2006; Cereda et al., 2016; Liu et al., 2017; Cholerton et al., 2018; Oltra et al., 2021). The benefits of continued use of object recognition memory tasks for these purposes include their proven utility for evaluating sex and sex hormone impacts in rodent models of PD (Luine, 2015; Costa et al., 2020; Lima et al., 2021; Pinizzotto et al., 2022). This along with the undisputed value of these tasks in identifying candidate neural substrates (Dere et al., 2007; Brown et al., 2012; Aggleton and Nelson, 2020; Barker and Warburton, 2020b; Chao et al., 2020) could ultimately help resolve points of common pathophysiological ground that render object recognition memories vulnerable not only in PD but also in other neurodegenerative disorders including Alzheimer's disease and schizophrenia (Grayson et al., 2015).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This animal study was reviewed and approved by IACUC, Stony Brook University.

AUTHOR CONTRIBUTIONS

CP: participated in all phases of animal testing, data collection, and data analyses, assisted co-authors in their responsibilities and primary responsibilities for creating figures and assisted in writing the manuscript. KD: primary responsibility for data analysis for Elevated Plus Maze testing, assisted in creating figures and writing of the manuscript. OA: primary responsibility for data analysis for Novel Object Location testing, assisted in creating figures and writing of the manuscript. RC: primary responsibility for behavioral testing and data analysis for Grip Strength and Tapered Balance Beam testing, assisted in creating figures and writing of the manuscript. KM: assisted in data analysis for Novel Object Recognition testing, assisted in creating figures and writing of the manuscript. MK: assisted in all phases of animal testing, data collection, data analyses, and creating figures, primary responsibility for writing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.951268/full#supplementary-material

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On the effect of hippocampal c-Jun N-terminal kinase inhibition on object recognition memory

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c-Jun N-terminal kinase (JNK) phosphorylates the transcription factor c-Jun in response to stress stimuli and contributes to both hippocampal synaptic plasticity and memory processing in mammals. Object recognition memory (ORM) is essential for remembering facts and events. In rodents, ORM consolidation and reconsolidation require a functional hippocampus. However, the possible involvement of hippocampal JNK on ORM processing has not yet been studied. Here we show that when injected into dorsal CA1 5 min, but not 6 h, after training adult male rats in the novel object recognition learning task, the JNK inhibitor SP600125 impaired ORM for at least 7 days without affecting exploratory activity, short-term ORM retention, or the functional integrity of the hippocampus. SP600125 did not hinder ORM retention when given in CA1 after a memory reactivation session carried out 24 h post-training in the presence of the same two objects presented during the training session, but caused time-dependent amnesia when one of the objects presented at training was replaced by a different but behaviorally equivalent novel one. Taken together, our results indicate that hippocampal JNK activity is necessary for ORM consolidation and reconsolidation but not for ORM recall or short-term retention.

KEYWORDS

consolidation, reconsolidation, recall, amnesia, hippocampus, SP600125

Introduction

c-Jun N-terminal kinases (JNKs) are a group of 46–55 kDa stress-responsive protein kinases encoded by the JNK1, JNK2, and JNK3 genes that belong to the mitogen-activated protein kinase family. Originally identified as the kinase activity that phosphorylates the transcription factor c-Jun, it is now clear that JNK also couples

cytokines- and growth factors-signaling to other nuclear and non-nuclear effectors, including the transcription factors ATF2, STAT3, and ELK1, the adaptor protein paxillin, the mitochondrial membrane protein BCL-2, and the protein kinases Akt and p90RSK, to regulate cell growth, differentiation, and apoptosis. In the brain, aside from its well-described participation in axodendritic morphogenesis (Komulainen et al., 2020), JNK signaling influences the pathogenesis of Alzheimer's disease (AD; Yarza et al., 2016), a progressive neurodegenerative illness that results in the loss of cognitive functioning. In fact, JNK seems to play important roles in synaptic plasticity and non-declarative memory. In this respect, mutant mice expressing an unphosphorylable c-Jun isoform show impaired hippocampal long-term potentiation (LTP; Seo et al., 2012), whereas pharmacological inhibition of hippocampal JNK enhances short-term memory and paired pulse facilitation and rescues stress-induced contextual fear conditioning from amnesia but blocks long-term fear-motivated avoidance memory consolidation, recall, and extinction (Bevilaqua et al., 2003, 2007; Li et al., 2007; Sherrin et al., 2010). However, it is currently unknown whether JNK is also involved in episodic memory, the type of declarative memory affected early in AD (Bäckman et al., 2001). Object recognition memory (ORM) allows animals to identify familiar items and is essential for remembering episodic information (Cole et al., 2019). In rats, ORM consolidation requires the functional integrity of several brain structures (Rossato et al., 2013), including the hippocampus (Clarke et al., 2010; Furini et al., 2010; ILL-Raga et al., 2013). The hippocampus also participates in ORM reconsolidation, a protein synthesis-dependent process that restabilizes and updates consolidated ORMs destabilized when recalled in the presence of a novel object (Rossato et al., 2007; Radiske et al., 2017; Gonzalez et al., 2021, 2022). Here, we analyzed whether hippocampal JNK is necessary for ORM consolidation and reconsolidation by assessing the effect on retention of the intra-dorsal CA1 administration of SP600125, a potent, cell-permeable, selective, and reversible ATP-competitive inhibitor of JNK (Bennett et al., 2001; Ennis et al., 2005) that does not affect other kinases or signaling pathways presently known to be important for the consolidation, recall, or reconsolidation of ORM in rats.

Materials and methods

Subjects

All experiments were performed during the light phase of the daylight cycle in agreement with the National Institutes of Health for the Care and Use of Laboratory Animals and the local institutional ethics committee [Comissão de Ética no Uso de Animais (CEUA) and UFRN] recommendations. We used a total of 198 adult male Wistar rats (3 months old; 300–350 g). They were housed in groups of five per cage and kept at 23°C in the institutional vivarium on a 12 h lights on/off schedule (lights on at 6:00 a.m.) with *ad libitum* access to food and water.

Stereotaxic surgery

Rats were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg) and bilaterally implanted with 22-gauge stainless steel cannula guides aimed to the CA1 region of the dorsal hippocampus (AP −4.2; LL, ±3.0; DV, −3.0). Stereotaxic coordinates were taken from Paxinos and Watson (2007). Rats received meloxicam (0.2 mg/kg) at the end of the surgical procedures and were allowed to recover for 7 days.

Drugs and injection procedures

SP600125 was obtained from Sigma-Aldrich (São Paulo, Brazil), dissolved in DMSO upon arrival, aliquoted, stored at −20°C and diluted to working concentration in sterile saline (0.9%) on the day of the experiment. For drug delivery, injection cannulas were fitted into the guides and injections (1 μl/side at 0.5 μl/min) carried out using a Hamilton syringe coupled to an infusion pump. The injection cannulas were left in place for 1 minute to minimize backflow. An equal volume of 0.1% DMSO in sterile saline was used as vehicle (VEH) control.

Novel object recognition task

Novel object recognition training and testing was conducted in a gray plywood open-field arena (60 cm × 60 cm × 60 cm) placed in a dim-light illuminated room acclimatized at 23–24°C, as described (Myskiw et al., 2008; Rossato et al., 2015). Briefly, rats were handled and allowed to explore the training arena in the absence of objects for 20 min/day during 4 days (habituation sessions). Twenty-four hours after the last habituation session, rats were exposed to two identical copies of the same novel object (object A) for 5 min in the training arena to induce ORM formation. To reactivate ORM, 24 h after training animals were re-exposed to familiar object A alongside novel object B in the training arena for 5 min. ORM retention was assessed only once per animal in a test session carried out 3 h, 24 h, or 7 days after training or reactivation. During the retention test, rats were exposed to familiar object A along with novel object C for 5 min. One hour before the experimental sessions, rats were transported from the vivarium to the experimental anteroom. From there, each rat was individually brought to the experiment room in a transport cage. At the end of each session, rats were returned to the experimental anteroom where they stayed for one additional hour before being transferred back to the vivarium. Objects were made of metal, glass, or

TABLE 1 Naive adult male Wistar rats display no innate preference for any of the objects utilized in the novel object recognition (NOR) task.

	Object exploration time (s)					
Object pair	**Object 1**	**Object 2**	**Total**	**DI**	***p***	***n***
A–A	30.98 ± 3.86	27.17 ± 3.21	58.15 ± 6.24	−0.06	0.264	11
A–B	28.58 ± 3.33	29.00 ± 3.86	57.58 ± 6.56	0.002	0.971	10
A–C	24.74 ± 2.50	24.19 ± 3.59	49.64 ± 7.44	−0.03	0.706	10
B–C	24.74 ± 2.50	26.33 ± 4.06	51.07 ± 6.00	−0.02	0.780	10

The table shows mean exploration time and DI ± SEM for naive animals during spontaneous object exploration in the training session of the NOR task. Total exploration time did not differ between objects pairs [$F(3,37) = 0.445$, $p = 0.7223$]. Discrimination indexes (DIs) are shown. p in one-sample Student's t test with theoretical mean = 0.

TABLE 2 Adult male Wistar rats trained in the novel object recognition (NOR) task discriminate between novel and familiar objects throughout the entire retention test session.

	1st min	**2nd min**	**3rd min**	**4th min**	**5th min**
DI	0.19 ± 0.08	0.20 ± 0.07	0.23 ± 0.04	0.20 ± 0.06	0.21 ± 0.08
p	0.0411	0.0167	0.0001	0.0112	0.0275
Object exploration time (s)	18.80 ± 2.18	16.27 ± 1.96	15.55 ± 2.03	15.51 ± 1.75	14.27 ± 1.49

The table shows mean ± SEM, discrimination index (DI), and total exploration time for each consecutive minute of a 5-min-long object recognition memory (ORM) retention test session in the presence of familiar object A and novel object C performed 24 h after NOR training in the presence of two identical novel objects A. p in one-sample Student's t test with theoretical mean = 0 ($n = 11$).

glazed ceramic and had no significance for the rats, which showed no innate preference for any of them (Table 1). The open-field arena and the objects were cleaned with 50% ethanol before each trial to ensure absence of olfactory cues. Object exploration was defined as sniffing and touching the objects with the muzzle and/or forepaws. Sitting on or turning around the objects was not considered exploratory behavior. A digital video camera fixed above the open-field arena was used for tracking the position and behavior of the rats. Video data were acquired at 30 frames/s and analyzed using the ObjectScan system (CleverSys). The discrimination index (DI) was calculated as follows: (time exploring novel object–time exploring familiar object)/total object exploration time, considering data from the 5 min session (Rossato et al., 2013). Naive rats discriminated between novel and familiar objects throughout the retention test session (Table 2). DI varied between −1 and +1; positive DI scores indicate preference for the novel object, whereas DI scores close to zero suggests absence of discrimination. Animals were excluded from data analysis when total exploration time during training, reactivation, or test sessions was less than 20 s (3 animals). We also excluded two animals that did not show object preference during reactivation session (RA).

Step-down inhibitory avoidance task

Inhibitory avoidance training was carried out as previously described (Rossato et al., 2006; Radiske et al., 2015). The IA training chamber was made of Plexiglas (50 cm × 25 cm × 25 cm) and contained an elevated wooden platform (5 cm × 8 cm × 25 cm) positioned at its left end. The floor of the chamber was a grid of bronze bars connected to a shock generator. At the beginning of the training session, animals were placed on the wooden platform and received a scrambled footshock (0.4 mA for 2 s) immediately after they stepped down to the grid. IA memory retention was evaluated 24 h after training by placing the animals on the training chamber platform and measuring their latency to step down. The test session finished when the animals stepped down to the grid or after 300 s, whatever happened first.

Data analysis

Statistical analyses were performed using GraphPad Prism 8 software. Significance was set at $p < 0.05$. NOR data were analyzed using one-sample t test with theoretical mean = 0 or two-way ANOVA followed by Bonferroni's multiple comparisons, as appropriate. IA data were analyzed using Mann–Whitney U test.

Results

Firstly, we examined whether hippocampal JNK inhibition affects ORM consolidation. To do that, we implanted adult male Wistar rats with guide cannulas aimed to the CA1 region of the dorsal hippocampus and trained them in the NOR task, an incidental episodic-like learning paradigm based on the rodents' innate preference for novelty (Ennaceur and Delacour, 1988; Clarke et al., 2008) involving exposure to two identical novel stimuli objects A in a familiar open field arena. Five minutes

or 6 h after training, animals received bilateral intra-dorsal CA1 injections (1 μl) of VEH (0.1% DMSO in sterile saline) or the JNK inhibitor SP600125 (20 μM; Bevilaqua et al., 2003, 2007), and 24 h post-training were exposed to one copy of familiar object A alongside a novel object B for 5 min to evaluate object A memory retention (**Figure 1A**). As can be seen in **Figure 1B**, animals that were given VEH discriminated novel object B from familiar object A during the retention test session regardless of the time elapsed between the training session and the moment of the injections. However, animals that received SP600125 5 min after training, but not 6 h thereafter, were unable to discriminate between objects A and B [**Figure 1B**; $F(1,40) = 5.802$, $p = 0.0207$ for treatment; $F(1,40) = 5.598$, $p = 0.0229$ for injection time, and $F(1,40) = 4.251$, $p = 0.0458$ for interaction; $t(40) = 3.131$, $p < 0.05$ for VEH 5 min vs. SP 5 min, $t(40) = 3.376$, $p < 0.01$ for VEH 6 h vs. SP 5 min, and $t(40) = 3.161$, $p < 0.05$ for SP 5 min vs. SP 6 h in Bonferroni's multiple comparisons test after two-way ANOVA]. SP600125 did not affect total distance traveled (**Figure 1C**), total exploration time (**Figure 1D**), or the total number of exploration events during the test session (**Figure 1F**). See **Figure 1E** for an illustration showing the position of injection cannulas in animals that received VEH or SP600125 5 min after training. Rats rendered amnestic with SP600125 were able to acquire and recall ORM upon retraining (**Figure 1G**) as well as to learn and express a fear-motivated avoidance response (**Figure 1H**) when trained in a step-down IA task (Alonso et al., 2005; Kerr et al., 2005; Bekinschtein et al., 2007), which also requires the functional integrity of the hippocampal formation (Bernabeu et al., 1995; Cammarota et al., 1998; Paratcha et al., 2000; da Silva et al., 2006; Katche et al., 2010). The amnesia caused by SP600125 lasted for at least 7 days [**Figure 1I**; $F(1,30) = 7.54$, $p = 0.0101$ for treatment, $F(1,30) = 7.235$, $p = 0.0116$ for injection time, and $F(1,30) = 4.871$, $p = 0.0351$ for interaction; $t(30) = 3.569$, $p < 0.01$ for VEH 5 min vs. SP 5 min, $t(30) = 3.844$, $p < 0.01$ for VEH 6 h vs. SP 5 min, and $t(30) = 3.502$, $p < 0.01$ for SP 5 min vs. SP 6 h in Bonferroni's multiple comparisons test after two-way ANOVA], but was not observed when ORM retention was assessed 3 h post-training (**Figure 1J**). The hippocampus is engaged in ORM reconsolidation in the NOR task only when the memory of the familiar object is reactivated in the presence of a novel one (Gonzalez et al., 2019; Rossato et al., 2019). Therefore, to analyze the possible participation of hippocampal JNK on ORM reconsolidation, 24 h post-training NOR-trained rats were re-exposed for 5 min to one copy of familiar object A alongside novel object B to reactivate the memory for object A and induce its hippocampus-dependent reconsolidation. Five minutes post-reactivation, or 6 h thereafter, animals received bilateral intra-CA1 injections of VEH or SP600125 (20 μM). Retention of the memory for object A was assessed 24 h afterward by exposing the animals to one copy of this object alongside novel object C (**Figure 2A**). Rats that received VEH or SP600125 6 h after object A memory reactivation discriminated this object from object C during the retention test session; animals that were given VEH 5 min after object A memory reactivation also remembered it 24 h later, but those given SP600125 failed to do so [**Figure 2B**; $F(1,37) = 4.573$, $p = 0.00391$ for treatment, $F(1,37) = 4.573$, $p = 0.0391$ for injection time, and $F(1,37) = 9.465$, $p = 0.0039$ for interaction; $t(37) = 3.825$, $p < 0.01$ for VEH 5 min vs. SP 5 min, $t(37) = 3.046$, $p < 0.05$ for VEH 6 h vs. SP 5 min, and $t(37) = 3.825$, $p < 0.01$ for SP 5 min vs. SP 6 h in Bonferroni's multiple comparisons test after two-way ANOVA]. Post-reactivation intra-CA1 SP600125 administration did not affect total exploration time (**Figure 2C**), or total distance traveled (**Figure 2D**) during the test session. As expected, neither VEH nor SP600125 had any effect on retention when injected in dorsal CA1 5 min or 6 h after submitting animals to an ORM RA in the presence of two copies of object A (**Figure 2E**). Pre-test intra-CA1 SP600125 administration did not impair ORM recall, but hampered object A memory retention during a second test session carried out 24 h after the first one in the presence of object A and novel object C [**Figure 2F**; $F(1,40) = 11.60$, $p = 0.0015$ for treatment, $F(1,40) = 4.912$, $p = 0.0324$ for injection time, and $F(1,40) = 6.327$, $p = 0.016$ for interaction; VEH-Test 1 vs. SP-Test 2: $t(40) = 3.975$, $p < 0.01$, VEH-Test 2 vs. SP-Test 2: $t(40) = 3.346$, $p < 0.05$, and SP-Test 1 vs. SP-Test 2: $t(40) = 4.187$, $p < 0.001$ in Bonferroni's multiple comparisons test after two-way ANOVA]. **Table 3** shows statistics for control experiments.

Discussion

Previously, we showed that ORM consolidation and reconsolidation after recall in the presence of a novel object require *de novo* protein synthesis in the hippocampus (Rossato et al., 2007; Myskiw et al., 2008). Here, we corroborated that the hippocampus is necessary for ORM consolidation, confirmed that ORM reactivation in the presence of a novel object induces hippocampus-dependent reconsolidation, and demonstrated that hippocampal JNK is necessary for these two processes. We also presented evidence showing that short-term ORM does not require JNK activity in dorsal CA1, which is not surprising given that short-term ORM does not appear to involve the hippocampal formation (Cohen et al., 2013). Our results can be unambiguously interpreted as due to the inhibitory action of SP600125 on JNK. Indeed, SP600125 hindered ORM retention when injected into dorsal CA1 5 min, but not 6 h after NOR training or ORM recall in the presence of a novel object, which demonstrates that the amnestic effect of this drug was time-dependent and therefore not due to impairment of hippocampal functionality. This claim is further supported by data showing that pre-test intra CA1 injection of SP600125 did not affect ORM memory recall, which requires the normal functionality of the hippocampal formation (Rossato et al., 2019), but hindered subsequent retention, and that animals rendered amnestic for ORM with SP600125 were

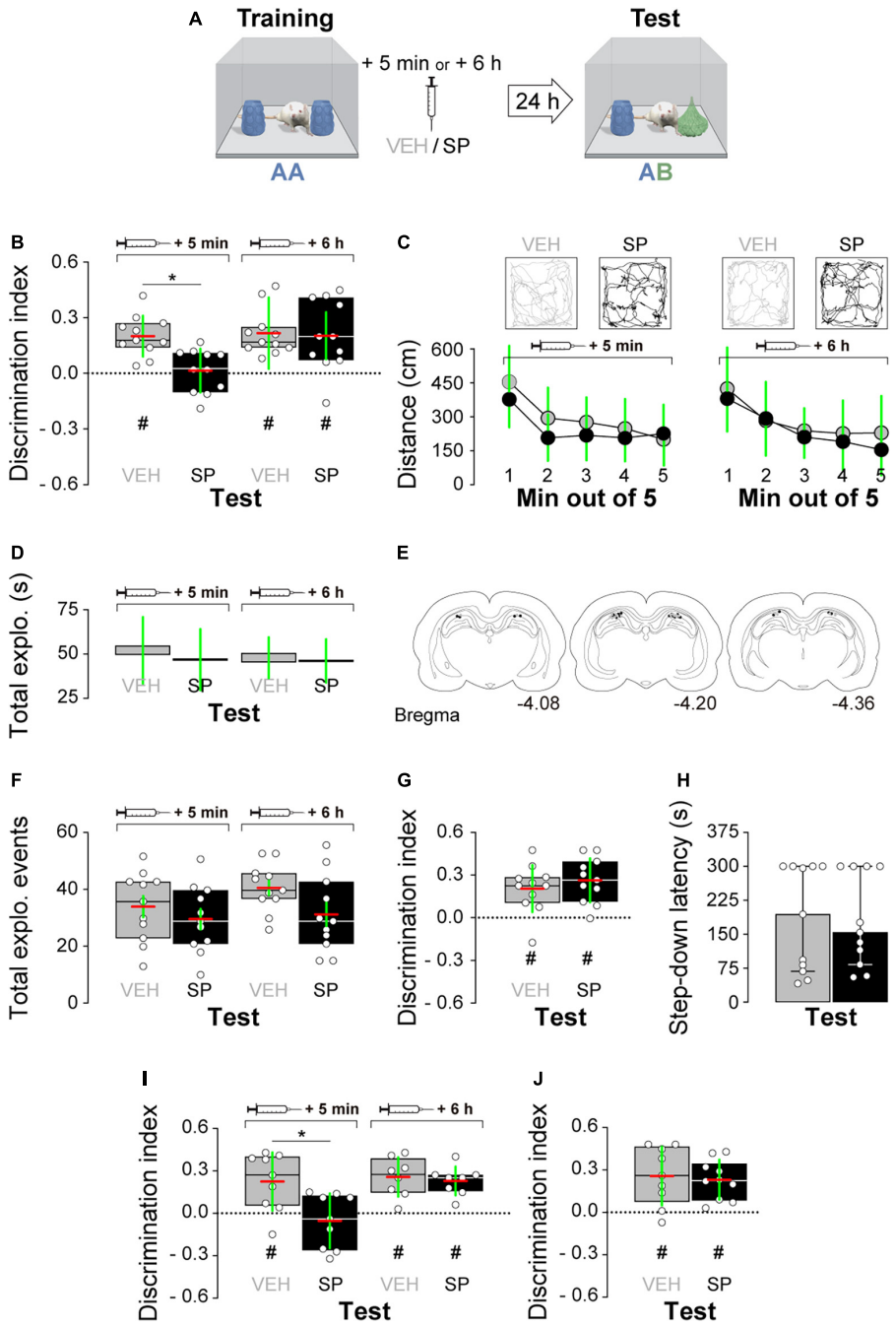

FIGURE 1
(A) Experimental protocol. **(B)** Rats trained in the novel object recognition (NOR) task using two copies of object A received bilateral intra-dorsal CA1 injections of SP600125 (SP; 20 μM; 1 μl/side) or vehicle (VEH; 0.1% DMSO in sterile saline) 5 min or 6 h post-training. Object A memory retention was assessed 24 h later (test) in the presence of familiar object A and novel object B. **(C**, top**)** Representative trajectory for VEH and SP-treated animals during Test. **(C**, bottom**)** Mean distance traveled during test for VEH and SP-treated animals. **(D)** Total object exploration time during Test. **(E)** Illustration showing cannula placement for animals that received VEH or SP 5 min after training. **(F)** Number of exploration events during Test. **(G)** Animals that received SP or VEH 5 min after training were retrained in NOR using a different pair of novel stimuli objects. Object recognition memory (ORM) retention was evaluated 24 h thereafter. **(H)** Animals that received SP or VEH 5 min after NOR training were trained in the inhibitory avoidance (IA) task. IA memory retention was evaluated 24 h thereafter. **(I)** Rats were treated as in panel **(A)** except that the retention test was performed 7 days post-training. **(J)** Rats were treated as in panel **(A)** except that the retention test was performed 3 h post-training. Discrimination index (DI) data are expressed as median (black or white horizontal lines) ± interquartile range (boxplots) and as mean (red horizontal line) ± SD (green vertical line). Dashed lines represent chance level. Total object exploration and distance traveled data are presented as mean ± SD. IA data are expressed as median ± interquartile range. n = 8–12 animals per group; $^{\#}p < 0.05$ in one-sample Student's t-test with theoretical mean = 0; and $^{*}p < 0.05$ in Bonferroni's multiple-comparison test after two-way ANOVA.

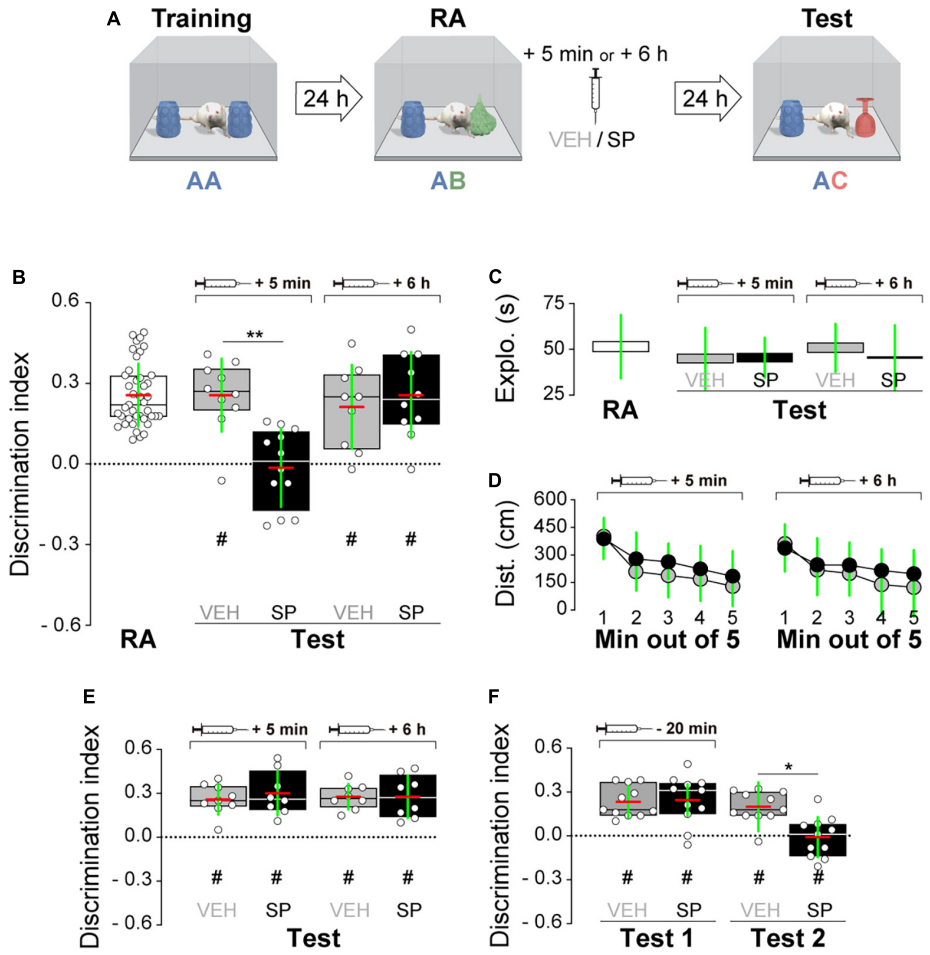

FIGURE 2

(A) Experimental protocol. **(B)** Rats were trained in the novel object recognition (NOR) task using two copies of object A and 24 h later they were submitted to an object recognition memory (ORM) reactivation session (RA) in the presence of familiar object A and novel object B. Five min or 6 h after RA, rats received bilateral intra-dorsal CA1 injections of SP600125 (SP; 20 μM; 1 μl/side) or vehicle (VEH; (0.1% DMSO in sterile saline). One day later rats were exposed to familiar object A and novel object C to evaluate ORM retention (Test). **(C)** Total exploration time during test. **(D)** Mean distance traveled during test for VEH and SP-treated animals. **(E)** Rats were treated as in panel **(A)**, except that RA occurred in the presence of two copies of familiar object A. **(F)** Rats were treated as in panel **(A)**, except that the animals received bilateral intra-CA1 injections of VEH or SP 20 min before Test 1. Discrimination index (DI) data are expressed as median (black or white horizontal lines) ± interquartile range (boxplots) and as mean (red horizontal line) ± SD (green vertical line). Dashed lines represent chance level. Total object exploration and distance traveled data are presented as mean ± SD. n = 8–12 animals per group; $^{\#}p < 0.05$ in one-sample Student's t-test with theoretical mean = 0; and $^{*}p < 0.05$ and $^{**}p < 0.01$ in Bonferroni's multiple-comparison test after two-way ANOVA.

later able to acquire and express ORM as well as a fear-motivated hippocampus-dependent IA response. Moreover, our experiments also indicate that the amnesic action of SP600125 cannot be attributed to a delayed effect on performance since this drug did not affect total exploration time, total distance travel, or the total number of exploration events during the retention test. JNK inhibitors have a deleterious effect on the consolidation of different hippocampus-dependent memories, including avoidance and extinction memories (Bevilaqua et al., 2003, 2007). These kinases may contribute to the consolidation process in several ways. They adjust the threshold for the induction of long-term synaptic plasticity, modulate neuronal excitability in a bi-directional manner through phosphorylation of AMPAR, and contribute to dendritic spine morphology and density in the hippocampus (Thomas et al., 2008; Komulainen et al., 2020). Moreover, JNK regulates synaptic transmission by controlling the synaptic levels of PSD-95 and thus, the internalization and reinsertion of AMPAR from and to the postsynaptic membrane (Kim et al., 2007), which are necessary steps for ORM destabilization and reconsolidation, respectively (Rossato et al., 2019). Prior work from our group shows that ORM consolidation and reconsolidation are associated with a late period of synaptic enhancement in the dorsal hippocampus (Clarke et al., 2010), and that gene expression and *de novo* protein synthesis in dorsal CA1 are necessary up to 3 h after training or recall for stabilizing new and updated memories

TABLE 3 Detailed statistics for control experiments.

Figures	Statistical method	*n*	Statistical details	
1C	Two-way ANOVA for infusion 5 min post-training	VEH: $n = 11$ SP: $n = 11$	Time vs. treatment: $F(4,80) = 1.49$ Treatment: $F(1,20) = 2.258$	$P = 0.2132$ $P = 0.1485$
	Two-way ANOVA for infusion 6 h post-training	VEH: $n = 11$ SP: $n = 11$	Time vs. treatment: $F(4,80) = 0.8701$ Treatment: $F(1,20) = 0.8256$	$P = 0.4857$ $P = 0.3744$
1D	Two-way ANOVA	VEH 5 min: $n = 11$ SP 5 min: $n = 11$ VEH 6 h: $n = 11$ SP 6 h: $n = 11$	Interaction: $F(1,40) = 0.1265$ Infusion time: $F(1,40) = 0.5847$ Treatment: $F(1,40) = 0.2946$	$P = 0.7240$ $P = 0.449$ $P = 0.5903$
1F	Two-way ANOVA	VEH 5 min: $n = 11$ SP 5 min: $n = 11$ VEH 6 h: $n = 11$ SP 6 h: $n = 11$	Interaction: $F(1,40) = 0.5205$ Infusion time: $F(1,40) = 3.822$ Treatment: $F(1,40) = 1.399$	$P = 0.4748$ $P = 0.0576$ $P = 0.2439$
1G	Unpaired *t* test	VEH: $n = 11$ SP: $n = 11$	$t(20) = 0.8229$	$P = 0.4203$
1H	Mann–Whitney test	VEH: $n = 11$ SP: $n = 11$	$U = 57$	$P = 0.8327$
1J	Unpaired *t* test	VEH: $n = 9$ SP: $n = 10$	$t(17) = 0.3729$	$P = 0.7138$
2C	Two-way ANOVA	VEH 5 min: $n = 10$ SP 5 min: $n = 12$ VEH 6 h: $n = 9$ SP 6 h: $n = 10$	Interaction: $F(1,37) = 0.3876$ Infusion time: $F(1,37) = 0.2829$ Treatment: $F(1,37) = 0.4121$	$P = 0.5374$ $P = 0.5980$ $P = 0.5249$
2D	Two-way ANOVA for infusion 5 min post-training	VEH: $n = 10$ SP: $n = 12$	Time vs. treatment $F(4,80) = 1.494$ Treatment F(1,20) = 2.602	$P = 0.2119$ $P = 0.1224$
	Two-way ANOVA for infusion 6 h post-training	VEH: $n = 9$ SP: $n = 10$	Time vs. treatment $F(4,68) = 1.096$ Treatment $F(1,17) = 1.95$	$P = 0.3655$ $P = 0.1806$
2E	Two-way ANOVA	VEH 5 min: $n = 9$ SP 5 min: $n = 8$ VEH 6 h: $n = 8$ SP 6 h: $n = 8$	Interaction: $F(1,29) = 0.2131$ Infusion time: $F(1,29) = 0.2395$ Treatment: $F(1,29) = 0.0035$	$P = 0.6478$ $P = 0.6282$ $P = 0.9534$

(Rossato et al., 2007, 2015; Radiske et al., 2017). In this regard, several transcription factors required for memory maintenance, such as AP-1 and Egr, are rapidly phosphorylated by JNK (Davis, 2000), and it has been reported that JNK knockdown mice show impaired early-LTP to late-LTP transition (Chen et al., 2005). Thus, it is possible that the amnesic effect of SP600125 on ORM is caused by deficient synaptic plasticity in the hippocampus. Given that consolidation and reconsolidation have differential molecular signatures (Bellfy and Kwapis, 2020), further research will be needed to determine whether the plastic changes underlying the storage of newly formed and updated ORM are regulated by different JNK isoforms, although the results we presented here suggest that, in both cases, the events mediated by this kinase occur no later than 6 h after training or recall, respectively. In the last decade, the use of pharmacological interventions as therapeutic co-adjuvants for the treatment of memory-related anxiety disorders has regained momentum. However, the limited number of mnemonically effective drugs

that are safe for human use remains one of the major problems of this approach. In this respect, our results are particularly interesting, because several JNK inhibitors are currently being tested in humans as anticancer, antidepressant, and anxiolytic drugs (Hollos et al., 2018; Wu et al., 2020).

Data availability statement

The original contributions presented in this study are included in this article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

This animal study was reviewed and approved by the CEUA–UFRN.

Author contributions

MC supervised the study. All authors conceived and carried out the experiments, analyzed the data, wrote the manuscript, and approved its final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Theta and gamma oscillations in the rat hippocampus support the discrimination of object displacement in a recognition memory task

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Episodic memory depends on the recollection of spatial and temporal aspects of past experiences in which the hippocampus plays a critical role. Studies on hippocampal lesions in rodents have shown that dentate gyrus (DG) and CA3 are necessary to detect object displacement in memory tasks. However, the understanding of real-time oscillatory activity underlying memory discrimination of subtle and pronounced displacements remains elusive. Here, we chronically implanted microelectrode arrays in adult male Wistar rats to record network oscillations from DG, CA3, and CA1 of the dorsal hippocampus while animals executed an object recognition task of high and low spatial displacement tests (HD: 108 cm, and LD: 54 cm, respectively). Behavioral analysis showed that the animals discriminate between stationary and displaced objects in the HD but not LD conditions. To investigate the hypothesis that theta and gamma oscillations in different areas of the hippocampus support discrimination processes in a recognition memory task, we compared epochs of object exploration between HD and LD conditions as well as displaced and stationary objects. We observed that object exploration epochs were accompanied by strong rhythmic activity in the theta frequency (6–12 Hz) band in the three hippocampal areas. Comparison between test conditions revealed higher theta band power and higher theta-gamma phase-amplitude coupling in the DG during HD than LD conditions. Similarly, direct comparison between displaced and stationary objects within the HD test

showed higher theta band power in CA3 during exploration of displaced objects. Moreover, the discrimination index between displaced and stationary objects directly correlated with CA1 gamma band power in epochs of object exploration. We thus conclude that theta and gamma oscillations in the dorsal hippocampus support the successful discrimination of object displacement in a recognition memory task.

KEYWORDS

hippocampus, local field potentials, recognition memory, spatial displacement of objects, pattern separation

Introduction

Living in a complex and dynamic world must require flexible memory systems capable of detecting subtle changes in the environment. The spatial and temporal aspects of past experiences are fundamental components in the recollection of episodic memories (Tulving, 2002; Eichenbaum et al., 2012). Once retrieved, previously acquired information is compared with current sensory inputs allowing the detection of contextual changes, which is critical to distinguish among similar episodic memories. This mnemonic process, named pattern separation, implements fine distinctions between similar patterns.

Researchers have been using spontaneous object exploration tasks as a tool to assess pattern separation and recognition memory in rodents, which exhibit a natural drive to detect and explore novelty in their environment (Barbosa and Silva, 2018; Wang et al., 2021). For instance, rats spend more time exploring a new object when compared to a familiar one in the novel object recognition task (Chao et al., 2020). A variant of this task, called novel object location task (NOL), evaluates spatial memory performance in rats. The animals are allowed to explore two equal objects in a familiar arena during the sample phase, and after a given interval one of these objects is moved to a new location. It is expected that rats spend more time exploring the displaced object relative to the stationary one (Ennaceur, 2010; Cohen and Stackman, 2015; Araujo et al., 2021). Hunsaker and Kesner (2008) have developed a NOL paradigm that sets different levels of object displacement as a tool to study spatial pattern separation. High displacements (HD) are expected to be more easily detectable by rats when compared to low displacement (LD) due to spatial interference between close object positions. The authors showed that hippocampal lesions in the dentate gyrus (DG) and CA3 impaired the discrimination of displaced objects. Specifically, DG lesions disrupted fine spatial discrimination and CA3 lesions affected global detection of alterations in the environment (Hunsaker and Kesner, 2008).

In parallel to lesion and behavioral studies, electrophysiological recordings have also implicated the hippocampus in recognition memory processes (Kemp and Manahan-Vaughan, 2004; França et al., 2015). Trimper et al. (2017) reported a critical role of the dorsal DG and CA3 slow gamma oscillations (30–60 Hz) during retrieval in an object recognition task. In particular, they have found the highest slow gamma power when rats explored novel objects, followed by familiar objects in swapped positions. The lowest level of slow gamma power occurred when rats explored familiar objects at the same locations, i.e., control groups. These results indicate a role for the DG and CA3 slow gamma activity in associative recognition memory for objects and their locations. Recently, Wang et al. (2021) detected an increase in ventral CA1 theta band power and in hippocampus-prefrontal theta band synchrony during the exploration of novel in opposition to familiar objects in a novel object recognition memory test. Additionally, a disturbed hippocampal-prefrontal connectivity performed by optogenetic silencing resulted in reduced theta synchrony and impaired novel object recognition. In contrast, Zheng et al. (2016) have found that fast gamma (60–100 Hz) band power in the dorsal CA1 was stronger during the retrieval as opposed to the sample phase when tested on a novel object in a novel location recognition task. Taken together, these studies reveal that hippocampal oscillations mediate mnemonic information during object recognition tasks, such as those involving changes in identity and positioning of objects.

Despite evidence pointing to the involvement of hippocampal theta and gamma rhythms in the discrimination of spatial object displacements, the role of different hippocampal areas remains elusive. Thus, here we postulate that theta and gamma oscillations in specific areas of the hippocampus support recognition memory while rats explore stationary and displaced objects. To test this hypothesis, we chronically implanted microelectrode arrays to simultaneously record from DG, CA3, and CA1 areas of the dorsal hippocampus of rats submitted to an object recognition task of high and low spatial displacements.

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Materials and methods

Animals

We used eight male *Wistar* rats (3 months old; ∼350 g) provided by the Biosciences Center Central Bioterium of the Federal University of Rio Grande do Norte. They were housed in a maximum number of four in standard cages (30 × 20 × 19 cm) on a 12 h/12 h light-dark cycle (lights on at 6 am) with food and water *ad libitum*. All the experiments were conducted on the light phase of the cycle. Experiments were approved by the Ethics Committee on the Use of Animals (CEUA/UFRN, permit n° 52/2016) and in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition (National Research Council [NRC], 2011).

Electrodes

We built microelectrode arrays (16 electrodes, 50 μm diameter Teflon-coated tungsten wires, California Fine Wire) designed to target the dorsal hippocampus of both hemispheres [−3.6 mm AP, ± 3.0 mm ML, according to Paxinos and Watson (2013)]. Electrodes were arranged in two 1 × 8 bundles with an inter-electrode lateral spacing of 250 μm, and an inter-electrode depth difference of 200 μm creating a stair design (**Figure 1A**). The eight microelectrodes were distributed across the laminar profile of the hippocampus (from 2.1 to 3.6 mm DV) to record electrophysiological signals from CA1, CA3, and DG from both hemispheres. The electrode impedance was reduced to ∼0.5 MOhms at 1 kHz in a gold solution with carbon nanotubes using NanoZ (Neuralynx) previously to surgery in accordance with previous studies (Ferguson et al., 2009).

Surgery

Animals were treated with atropine (0.04 mg/kg, s.c.), anesthetized with ketamine and xylazine (respectively, 100 mg/kg and 8 mg/kg, i.p.), and placed in a stereotaxic (Insight Equipamentos). Rectangular craniotomies were made to allow electrode insertion into the brain tissue. Two stainless steel screws soldered to a silver wire were implanted in the occipital cranial bone to provide ground and reference. Four additional stainless steel screws were positioned into the parietal and frontal cranial bones to provide mechanical support to the electrode arrays. Acrylic resin was used to cement the electrode array at the final target position. After surgery, animals were treated with anti-inflammatory (flunixin-meglumine at 2.5 mg/kg, i.p.), anti-biotic (enrofloxacin at 10 mg/kg, s.c.), and analgesic (paracetamol at 200 mg/kg, oral) for the following 3 days.

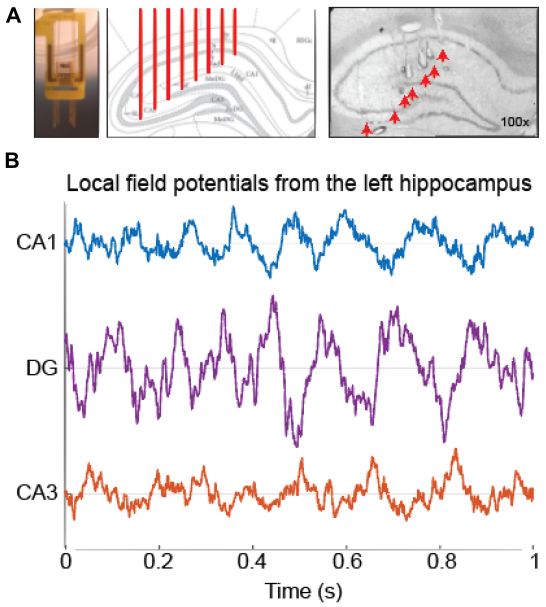

FIGURE 1
Histology and electrophysiological recordings. **(A)** Representative picture of: a 16-ch microelectrode array designed to bilaterally record from DG, CA3, and CA1 areas of the dorsal hippocampus (left); the targeted anatomical areas (3,6 mm posterior to Bregma; Paxinos and Watson, 2013) and electrode positions in the hippocampus (red vertical lines, middle); a representative 50-μm Nissl-stained coronal section of the left hemisphere of the dorsal hippocampus (right). Red arrows point to tissue lesions caused by electric currents individually applied at each electrode. **(B)** Representative examples of simultaneous recordings of hippocampal local field potentials. Traces on different colors show selected electrodes positioned in each hippocampal area: CA1 (blue), DG (purple), and CA3 (red). Note the phase reversal of the theta cycle between the DG and CA1/CA3.

Experimental protocol

After recovery, animals were allowed to a daily session of 30 min of acclimation in the experimental room before starting any procedure. During five consecutive days, rats were handled for 20 min in order to reduce stress related to the presence and physical contact with the experimenters. In the first two days, handling was performed in the homecage collectively, and in the following 3 days rats were handled individually. Additional handling sessions of 5 min were performed on task days. In the next 4 days, animals were habituated to the apparatus (the rest box used to hold animals during the inter-task intervals and the open field, see description below) for 10 min per day. No object was presented in the open field in the habituation sessions. See **Supplementary Figure 1** for a detailed schema of the experimental design.

The object recognition task was performed in an all-black circular open field (height 45 cm, diameter 118 cm) with four proximal cues on the arena walls and another four additional

distal cues on the walls of the experimental room. We used two copies of the same object made of glass or ceramic materials (see pictures of the objects in **Supplementary Figure 2**). Objects were positioned radially in the open field 4 cm distant from the walls. At trial start, animals were placed in the center of the open field facing the northern direction. During the 10-min intervals of the task, rats were placed on a rest box (height 45 cm, width 45 cm, length 45 cm) of white walls and a black floor.

Novel object location recognition task

To assess the behavioral correlates of spatial novelty detection we adapted a protocol from Hunsaker and Kesner (2008). The protocol consisted of one 5-min sample trial in which rats were presented to two identical objects and two 5-min test trials in which one of the objects was moved to a new place (**Figure 2A**). Spatial displacements were performed at large or at small distances. When the object was moved 108 cm from its previous position, the test was defined as a high spatial displacement (HD, as shown in **Figure 2A**). When the object was moved 54 cm from its previous position, the test was defined as a low spatial displacement (LD). Half of the animals were first exposed to HD followed by LD, while the other half had the exposure given in the opposite order. The spatial positions and type (ceramic or glass) of objects were randomized among animals.

Data collection

Continuous electrophysiological recordings were performed using a headstage preamplifier wired-coupled to a multi-channel recording system (RHA2116, Intan Technologies). Raw electrophysiological signals were filtered between 0.02 and 20 kHz and recorded at 30 kHz. Animal behavior was recorded in video by a high-definition digital camera positioned above the rest box and the open field apparatus (1080 × 720 pixels at 30 frames/s, Logitech C920). Video and electrophysiological recordings were synchronized by a microcontroller (Arduino Uno) and stored for posterior analysis.

Behavioral analysis

The video recordings were analyzed using the Ethowatcher software (Junior et al., 2012) by a researcher blinded to the experimental manipulations: the level of spatial displacement (HD vs. LD) and the label of the objects (stationary or displaced objects). We considered as object exploration time intervals when a rat faced an object at least 2 centimeters away from its snout for more than 1 s. In order to reduce locomotion-related modulation of hippocampal oscillations, the following analyses have only included behavioral epochs of object exploration in which the animals were clearly still and not running or walking. The total exploration time was then calculated as the sum of the time spent exploring each object cumulatively minute-by-minute within each 5-min session (sample, HD and LD tests), similarly to previous studies (Dix and Aggleton, 1999; Ameen-Ali et al., 2015; Araujo et al., 2021). In order to test novel object location recognition memory, we used a discrimination index that evaluates the animal's spontaneous preference for one of the objects. The discrimination index measure was calculated as the ratio between the time spent exploring the displaced object (tDO) minus the stationary object (tSO) and the sum of the time spent exploring both objects (tDO + tSO) in a cumulative minute-by-minute approach [(tDO − tSO)/(tDO + tSO)] (Ennaceur and Delacour, 1988; Inostroza et al., 2013; Cohen and Stackman, 2015). Positive discrimination index values indicate a preference for the displaced object, negative values indicate a preference for the stationary object and zero denotes no preference.

Electrophysiological analysis

Signal analyses were made using custom-made and built-in routines in MATLAB (MathWorks). At first, the raw electrophysiological signals from the 16 electrodes were downsampled from 20 to 1 kHz in order to obtain the local field potentials (LFP). To do that, we used the "resample" function from the Signal Processing Toolbox, which avoids aliasing effects. We then selected one electrode from each hippocampal area per hemisphere based on differences in the phase of theta oscillations, similarly to Scheffer-Teixeira et al. (2012). Specifically, we filtered the LFP in the theta (6–12 Hz) band using the "eegfilt" function from the EEGLAB Toolbox (Delorme and Makeig, 2004). We then calculated the Hilbert transform using the "hilbert" function from the Signal Processing Toolbox to obtain the instantaneous theta phase of each LFP, from which we calculated the theta phase difference between the most superficial electrode and all other electrodes from the same hemisphere. Since theta phase reversal is known to occur between CA1/CA3 areas and the DG close to the stratum radiatum and stratum lacunosum-moleculare (Brankack et al., 1993; Buzsáki, 2002; Csicsvari et al., 2003), we selected CA1 electrodes that showed the lowest phase difference, DG electrodes that showed the highest phase difference (i.e., phase reversal), and CA3 electrodes that showed the lowest phase difference relative to the theta phases exhibited by the most superficial electrode. Electrode positioning was confirmed by the profile of power in the theta and slow gamma (25–55 Hz) bands (**Supplementary Figure 3**), which peak at the hippocampal fissure and at the hilus of the DG, respectively

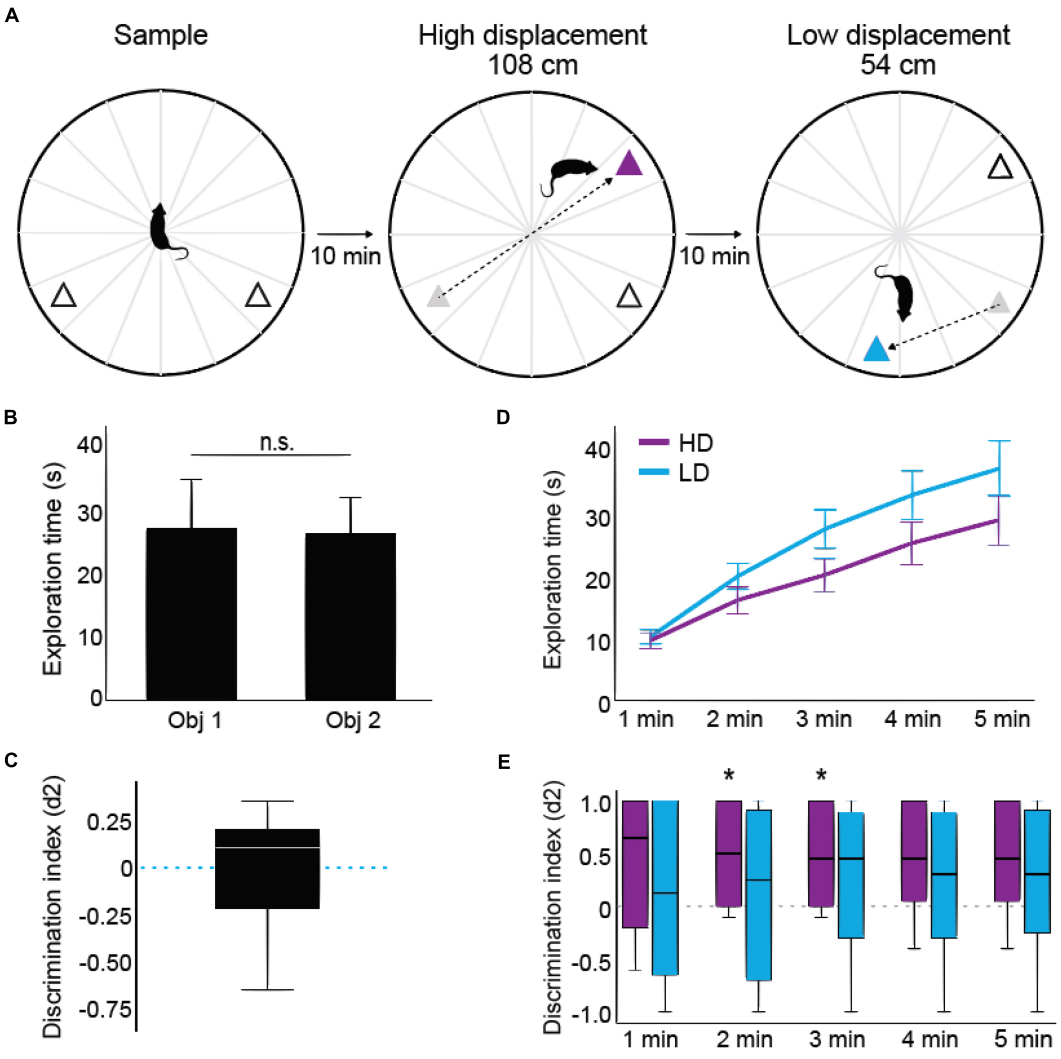

FIGURE 2
Experimental design and behavioral performance. **(A)** Schematic illustration of the object recognition task with high and low spatial displacement tests. In the sample phase, rats explored two identical objects (triangles) in a circular open field (118 cm diameter) for 5 min. In the high and low displacement tests, one object was spatially displaced by 108 cm and another by 54 cm, respectively. **(B)** Total exploration time of each object during the sample session. Bars represent means and error bars represent SEM. **(C)** Discrimination index among objects [(tDO − tSO)/(tDO + tSO)] calculated during the sample session. The whisker plot shows the distribution of discrimination index values, where the white line depicts median, black bars represent upper and lower quartiles, and error bars represent values outside the middle 50%. Dashed line depicts chance values (i.e., rats devoted the same time to exploring both objects). **(D)** Minute-by-minute cumulative analysis of the time of object exploration during the high and low spatial displacement tests (purple and cyan, respectively). Lines depict means and error bars represent SEM. **(E)** Minute-by-minute cumulative analysis of the discrimination index in HD and LD tests (purple and cyan, respectively). Black lines depict medians, bars represent upper and lower quartiles, and error bars represent values outside the middle 50%. Asterisks indicate $p < 0.05$ against zero (i.e., chance levels; Wilcoxon signed-rank test), $n = 8$ animals.

(Brankack et al., 1993; Bragin et al., 1995; Buzsáki, 2002; Csicsvari et al., 2003).

Next, LFP signals from epochs of object exploration were concatenated into a single continuous string of data for each area (Sabolek et al., 2009) and labeled according to each animal, object identity and displacement condition. Two experimental conditions were directly compared: (1) epochs of object exploration during both HD and LD test conditions, and (2) epochs of exploration of stationary and displaced objects within HD test condition since animals only discriminated between stationary and displaced objects in this test session.

We analyzed the power spectra at the theta (6–12 Hz), slow gamma (25–55 Hz), and fast gamma (65–110 Hz) band frequencies (Buzsáki and Draguhn, 2004; Zheng et al., 2016). We used the "spectrogram" function (0.5-s window, with 50% overlap) to obtain the time-frequency decomposition of LFP signals shown in **Figures 3**, **4**. We used the "pwelch" function (1-s window, with no overlap) to obtain the power spectral density

of LFPs. The power at a given frequency band was defined as the mean of the power spectral values within the band of interest. The power of each frequency band was then averaged across animals and test conditions to obtain the mean power of the group in HD and LD test conditions.

To evaluate the phase-amplitude cross-frequency coupling (CFC), the modulation index was calculated as previously described by Tort et al. (2008). Briefly, the modulation index for several frequency pairs of low-frequency "phase-modulating" and high-frequency "amplitude-modulated" components was evaluated. We first filtered spectral components of the LFPs in the theta and gamma bands. Phase bandwidth of 4 Hz at 0.5-Hz-steps were used to obtain the phases of theta oscillations between 5 and 10 Hz, and amplitude bandwidth of 10 Hz at 5-Hz-steps were used to obtain the gamma amplitude between 20 and 120 Hz. We next calculated the Hilbert transform to obtain the instantaneous phase of theta oscillations and the instantaneous amplitude of gamma oscillations. The modulation index was obtained for each electrode and experimental condition individually. To obtain the CFC between theta phases and a given subcomponent of the gamma frequency band, we averaged the modulation index within the slow and fast gamma band frequencies previously defined. We thus compared modulation index values between HD and LD conditions. No CFC analysis was performed to compare phase-amplitude modulation during the exploration of stationary and displaced objects due to short epochs of contact with objects for two animals, i.e., the total time of contact with one of the objects was lower than 1.5 s. Modulation index values were graphically expressed as color-coded plots (**Figure 5**), in which hot colors in the c-axis indicate that the phase-frequency in the x-axis modulates the amplitude-frequency in the y-axis.

In order to reduce variability among animals due to differences in electrode impedance, electrode position, and other factors, statistical comparisons of band power were performed after the normalization of individual data points (animal and condition) by the mean across conditions (Tort et al., 2009; Belchior et al., 2014; Furtunato et al., 2020). For instance, in the power spectral analysis (**Figures 3B**, **4B**), the theta band power in HD condition was divided by the mean power across HD and LD conditions Normalized HD = HD/[(HD + LD)/2]. The same normalization was applied to LD conditions before statistical comparison. Thus, the sum of power in HD and LD conditions after normalization must be equal to one.

Statistical analysis

MATLAB (MathWorks) and SPSS (v.26, IBM) were used for statistical analyses, and results were considered significant at an α level lower than 0.05. SPSS was used for behavioral analyses, while MATLAB was used for electrophysiological analyses and their correlations with behaviors. The Shapiro-Wilk test was used to analyze data normality in both behavioral and electrophysiological datasets. For behavioral analyses, the two-way ANOVA followed by the Sidak-Bonferroni *post-hoc* test was used to compare the total exploration time across time and conditions (minute-by-minute and high and low spatial displacement tests, respectively). The Wilcoxon signed-rank test was used to compare the discrimination indexes against chance levels (i.e., no preference: 0), and also to directly compare the discrimination indexes between conditions of HD and LD. The paired *t* test was used to compare band power values between exploration time in HD and LD test conditions and between exploration of objects in displaced and stationary conditions. The "corr" function was used to evaluate the Spearman's rank order correlation (rho) between the discrimination index and the power of theta, slow gamma and fast gamma bands. We used the GPower software to calculate Cohen's (d') effect size (Faul et al., 2009), in which we considered d' values > 0.8 as a large effect size.

Results

Rats discriminate between stationary and displaced objects in the high displacement test

Rats executed an object recognition task with high and low spatial displacement test (HD: 108 and LD: 54 cm; see **Figure 2A**). During the sample session, no significant difference was found neither in the total time exploring the two objects [**Figure 2B**, $t(7) = 0.154$, $p = 0.882$, $d' = 0.055$, paired *t* test] nor in the preference for specific objects [i.e., discrimination index against 0; **Figure 2C**, $t(7) = 0.077$, $p = 0.941$, $d' = 0.696$, Wilcoxon signed-rank test]. During the tests, the total exploration times in HD and LD conditions were not statistically different (**Figure 2D**). Two-way ANOVA, in a minute-by-minute cumulative analysis, detected no differences between the task conditions [HD x LD, $F(1,27) = 2.836$, $p = 0.104$, $d' = 0.323$] nor detected differences for factor interaction [$F(4,108) = 2.348$, $p = 0.120$, $d' = 0.294$], which suggests equivalent motivational drive to explore the objects in both HD and LD tests. Also, a direct comparison of the discrimination index in HD and LD conditions revealed no significant difference ($p = 0.089$, $d' = 0.287$, Wilcoxon signed-rank test). In spite of that, rats exhibited an exploration preference for the displaced object in opposition to the stationary object in the HD test, as shown by the discrimination index statistically higher than chance in minutes 2 (**Figure 2E**, $p = 0.048$; $d' = 1.005$) and 3 ($p = 0.048$; $d' = 0.971$); while minutes 1 ($p = 0.105$; $d' = 1.798$), 4 ($p = 0.061$; $d' = 0.825$), and 5 ($p = 0.061$; $d' = 0.825$) were not significantly

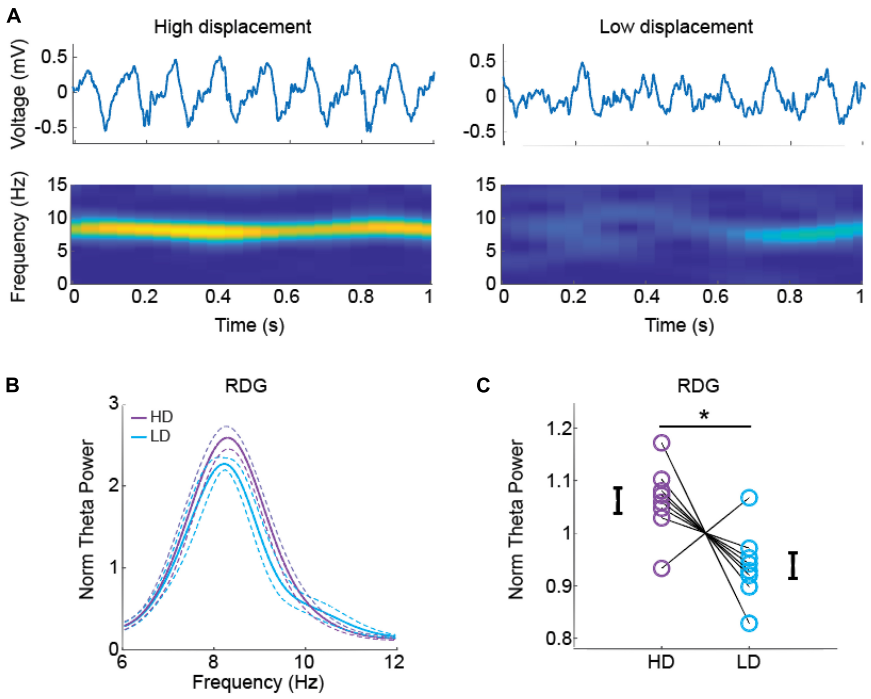

FIGURE 3
Theta oscillations in the dentate gyrus (DG) during object exploration in the high and low spatial displacement tests. **(A)** Representative raw local field potentials (LFP) (upper) and spectrograms (lower) from the right DG during 1-s of object exploration in the high and low spatial displacement tests. **(B)** Normalized average power spectra at the theta (6–12 Hz) band in the right DG during high and low spatial displacement tests (purple and cyan, respectively). Solid lines represent mean and dashed lines represent SEM. **(C)** Normalized mean theta power in right DG area at high and low spatial displacement tests. Purple and cyan circles represent normalized theta power from individual rats and error bars represent SEM. Asterisk indicates statistical significance in a paired *t* test, $n = 8$ animals.

different from chance (Wilcoxon signed-rank test against 0). In contrast, the discrimination index in LD tests was not statistically different from zero (see **Supplementary Table 1**). These behavioral results suggest that rats do discriminate between stationary and displaced objects in conditions of pronounced spatial changes, but not in smaller spatial change conditions.

In order to investigate whether oscillatory activity in the hippocampus is associated with the discrimination of stationary and displaced objects–either in small or pronounced spatial changes - we bilaterally recorded local field potentials from CA1, CA3, and DG areas using multielectrode arrays (**Figure 1A**, left and middle). Histological analysis confirmed electrode tip positions at the dorsal hippocampus (**Figure 1A**, right). We then used the theta phase reversion between LFP signals from DG and CA1/CA3 to select one electrode from each subfield in both hemispheres (**Supplementary Figure 3**). **Figure 1B** (and **Supplementary Figure 3**) shows representative raw local field potentials during 1-s of rhythmic activity in the theta (6–12 Hz) band obtained from the left hemisphere of the hippocampus. Panels C and D of the **Supplementary Figure 3** show the laminar profile of theta power and slow gamma power across the dorsal hippocampus.

Recognition memory was associated with higher theta power in the dentate gyrus

Subsequently, we next investigated whether there would be any differences in oscillatory LFP activity during the retrieval/test phase between the condition animals detected the displaced object (HD tests) and the condition animals did not discriminate between displaced and stationary objects (LD tests). Raw LFPs and spectral decompositions during object exploration epochs exhibited stronger theta rhythm in the HD test in comparison to the LD test (**Figure 3A**, left and right panels respectively). The group result shows that the normalized theta power in the right DG was also statistically higher in the HD test [**Figures 3B, C**, RDG, $t(7) = 2.576$, $p = 0.036$, $d' = 0.911$]; no significant difference was observed in the left DG. We found no statistical difference in theta power between HD and LD conditions neither in CA3 nor CA1 areas. We found no significant difference between HD and LD conditions neither for the slow gamma (25–55 Hz) nor fast gamma (65–110 Hz) bands. **Supplementary Table 2** shows statistical results for power spectra comparisons between HD and LD conditions in the theta, slow and fast gamma bands.

Theta-fast gamma phase-amplitude coupling in the dentate gyrus was higher in HD than LD tests

We also evaluated whether the theta phase modulates the amplitude of gamma oscillations during object contacts and whether it changes between different memory conditions in HD and LD tests. A representative example of theta-phase-associated gamma burst in the left DG is shown in **Figure 5A**. Comodulograms from the left DG show that in both HD and LD conditions the modulation index peaked at ∼80 Hz, within the fast gamma (65–110 Hz) band (**Figure 5B**). We found that the theta-fast gamma phase-amplitude coupling was significantly higher during the HD than LD tests in the left DG [**Figure 5C**, LDG $t(5) = 3.856$, $p = 0.012$, $d' = 1.074$; paired t test]. We found no significant difference in the theta-gamma modulation between HD and LD in other brain areas. **Supplementary Figure 4** shows comodulograms from individual rats and **Supplementary Table 3** shows statistical results according to brain areas and slow and fast gamma frequency bands.

Exploration of displaced objects was associated with higher theta power in CA3

To further investigate whether hippocampal rhythms are associated with the discrimination of objects, we compared LFPs during the exploration of displaced and stationary objects in the HD test. Spectral analysis revealed the presence of theta oscillations during exploration of both stationary and displaced objects (**Figure 4A**). Normalized theta band power in the left CA3 was higher during the exploration of displaced objects than stationary ones [**Figures 4B, C**, LCA3 $t(4) = 3.250$, $p = 0.031$, $d' = 1.181$, paired t test]. We found no significant differences between stationary and displaced objects in theta band power in the right CA3, nor in DG and CA1 of both hemispheres; we found no significant differences in the slow and fast gamma band power in none of the areas. **Supplementary Table 4** shows statistical results of spectral power according to brain areas and frequency bands.

The discrimination index was correlated with gamma band power in CA1

We next analyzed the relationship between the discrimination index and the power of hippocampal theta, slow and fast gamma oscillations during object exploration epochs. We found no significant relationship between the discrimination index and theta, slow or fast gamma band power in the LD tests. Nonetheless, the discrimination index positively correlated with the gamma band power in the right CA1 area. Both slow and fast gamma band power exhibited during object exploration were significantly correlated with the discrimination index (**Figures 6A, B**, rho = 0.829, $p = 0.016$; rho = 0.927, $p = 0.005$, respectively). No significant correlation was observed between the discrimination index and theta, slow or fast gamma in other brain areas (see **Supplementary Table 5**).

We also evaluated the correlation between the discrimination index and theta, slow and fast gamma band power specifically obtained during exploration of displaced and stationary objects within the HD tests. The discrimination index positively correlated with slow gamma power in the right CA1 exhibited during exploration of stationary objects (**Figure 6C**, rho = 1, $p = 0.016$). Moreover, the discrimination index was positively correlated with fast gamma band power in the right CA1 during exploration of the displaced object (**Figure 6D**, rho = 0.936, $p = 0.004$). Of note, the discrimination index was also inversely correlated with the theta band power in the right DG during the exploration of the stationary object (rho = −1, $p = 0.016$; **Supplementary Tables 6, 7**).

Discussion

We employed an object recognition task and multielectrode recordings from the rat hippocampus to investigate the electrophysiological correlates of the recognition memory for spatial displacements of objects by large and small distances. Our results show that rats do discriminate between stationary and displaced objects in conditions of pronounced displacement (HD, 108 cm) but not low displacement (LD, 56 cm), which allowed us to directly compare between different behavioral outcomes in the retrieval phase of the test. Spectral analysis of the LFP activity revealed (1) prominent theta oscillations during epochs of contact with the objects, (2) higher theta power in the right DG during HD than LD tests, (3) higher theta-gamma phase-amplitude coupling in the left DG during HD than LD tests. In addition, (4) contacts with displaced objects exhibited higher theta power in the left CA3 than stationary objects in the HD tests. Finally, (5) the discrimination index directly correlated with gamma band power in the right CA1 during object contacts, in which slow gamma oscillations related to exploration of stationary objects (i.e., memory retrieval) and fast gamma oscillations related to displaced objects (encoding). In all, these findings suggest that the theta and gamma oscillatory activity in the dorsal hippocampus is positively related to object discrimination in a recognition memory task.

Recent studies have used recognition memory tasks in rodents to investigate the discrimination of spatially displaced and stationary objects, as well as the underlying processing of memory encoding and retrieval in hippocampal circuits.

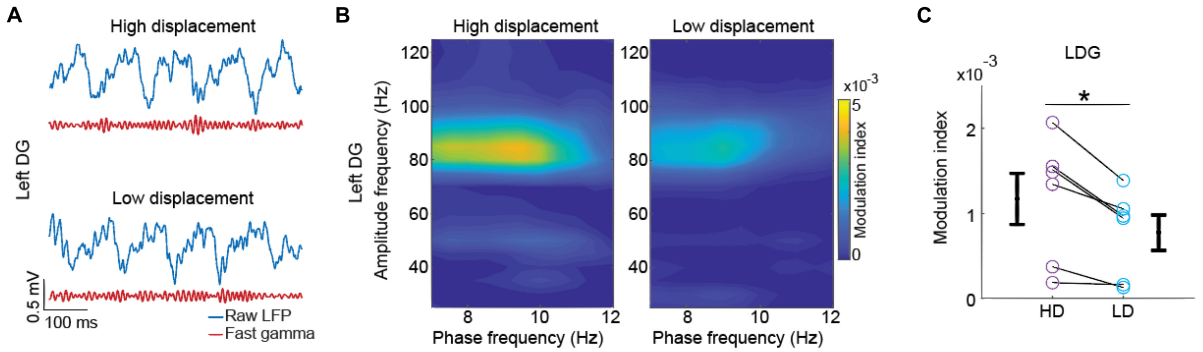

FIGURE 4
Phase-amplitude cross-frequency coupling between theta and fast gamma oscillations in the dentate gyrus during high displacement (HD) and low displacement (LD) tests. **(A)** Raw local field potentials (LFP) signals (blue) and their respective fast gamma-filtered components (red) obtained from the left DG during object exploration in the high and low spatial displacement tests (upper and lower, respectively). **(B)** Representative theta-gamma phase-amplitude modulation in the left DG during object exploration in high and low displacement tests (left and right, respectively). **(C)** Average modulation index between theta phases and fast gamma amplitude in the left DG during high and low displacement tests (purple and cyan, respectively). Circles represent modulation index for individual rats and error bars represent SEM. Asterisk indicates statistical significance in a paired *t* test, n = 6 animals.

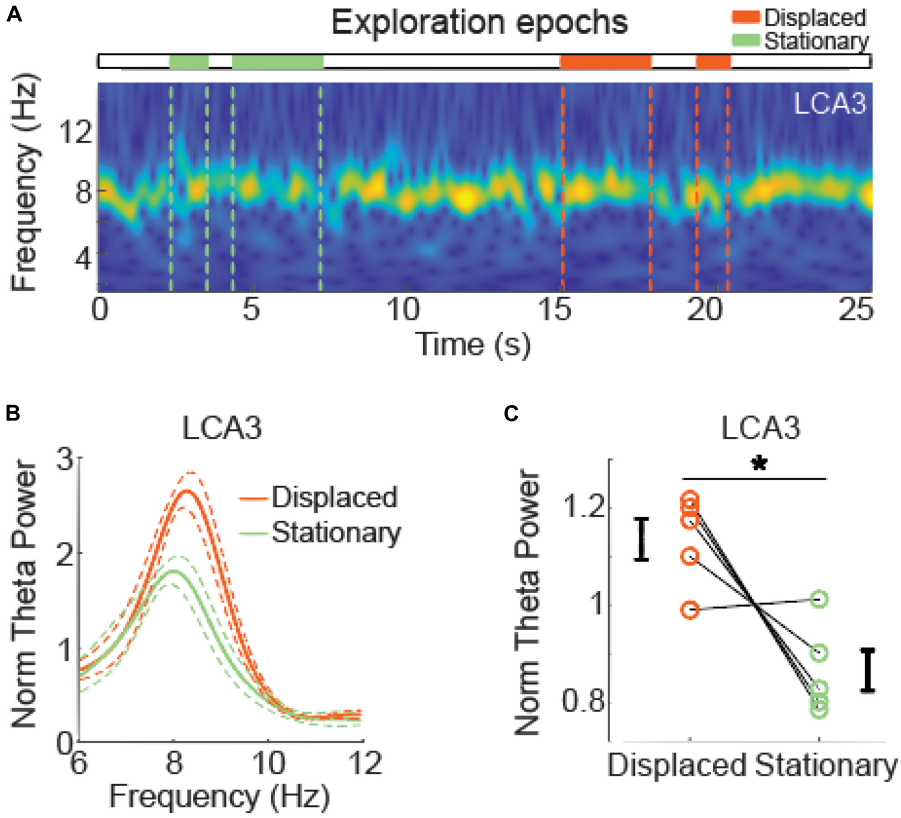

FIGURE 5
Theta oscillations in CA3 during exploration of displaced and stationary objects in the high displacements (HD) tests. **(A)** Representative spectrogram of local field potentials (LFP) signals from the left CA3 area during a high displacement test. Horizontal bars and vertical dashed lines depict time intervals of exploration of displaced and stationary objects (orange and green, respectively). **(B)** Normalized average power spectra at the theta (6–12 Hz) band in the left CA3 during exploration of displaced and stationary objects in HD tests. Solid lines represent mean and dashed lines represent SEM. **(C)** Normalized mean theta power in the left CA3 during exploration of displaced and stationary objects. Circles represent mean theta power of individual rats and error bars represent SEM. Asterisks represent statistical significance in a paired *t* test, $n = 5$ animals.

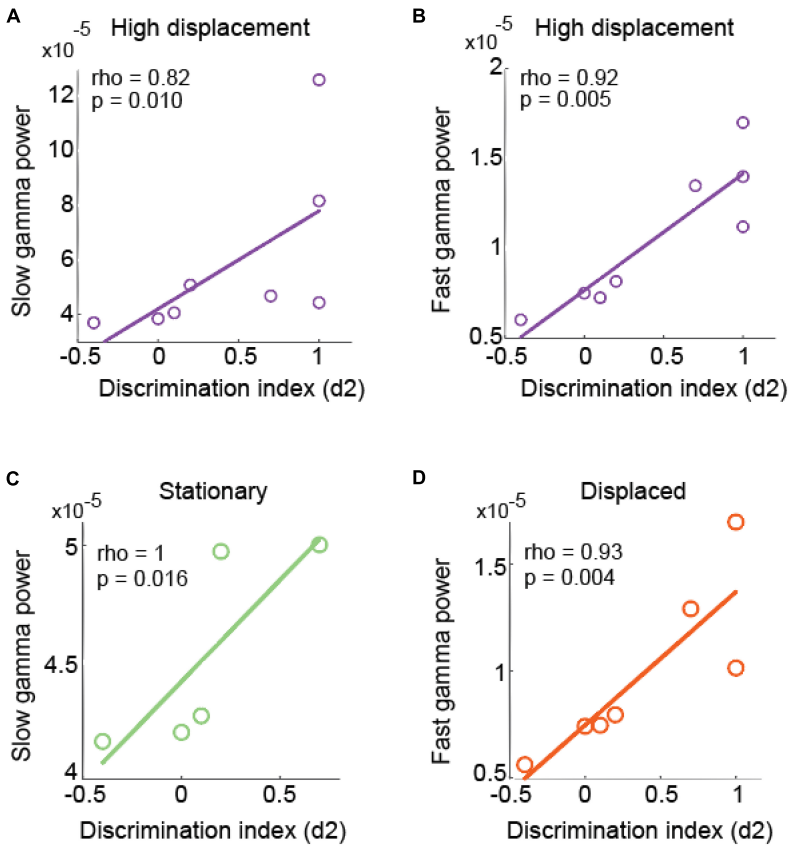

FIGURE 6
Relationship between the discrimination index and the power of slow and fast gamma bands in CA1 during exploration of displaced and stationary objects in high displacements (HD) tests. Relationship between the discrimination index and the power of slow gamma **(A)** and fast gamma **(B)** bands exhibited in the right CA1 during object exploration in HD tests. Circles represent individual animals, and the straight line depicts the linear relationship between variables. **(C)** Relationship between the power at slow gamma band exhibited during exploration of stationary (green) objects and the discrimination index in HD tests. **(D)** Relationship between the power at fast gamma band exhibited during exploration of displaced (orange) objects and the discrimination index in HD tests. Panels **(A–D)** represent 8, 5 and 7 animals, respectively.

Hunsaker and Kesner (2008) found that chemical lesions in the rat DG impairs the discrimination of previously encountered objects in conditions of low but not high spatial displacements, suggesting that the DG processing is critical to detect fine spatial displacements. In their experiment, sham-lesioned rats (control group) exhibited significant discrimination index scores in both high (108 cm) and low (56 cm) spatial displacement tests (Hunsaker and Kesner, 2008). Contrasting to that, we have found that rats were only capable of discriminating between stationary and displaced objects–i.e., discrimination indexes higher than chance - in large displacement conditions (108 cm). Such contrast allowed for the comparison between different memory outcomes and their underlying mechanisms.

Since the task protocols in both studies followed similar displacement conditions, we attribute this behavioral difference to divergences in the amount of sampling/test phases: while Hunsaker and Kesner (2008) used three sampling phases followed by one test, here we used only one sampling phase followed by two tests. It might be the case that task designs with multiple sample trials–as used by Hunsaker and Kesner (2008)–may facilitate the acquisition of memory for the spatial location of objects in fine displacement conditions. In addition, the two studies also differed in the strain of rats used: Hunsaker and Kesner (2008) used Long Evans rats and we used Wistar strain, which could also contribute to the observed variability (Andrews et al., 1995). Nevertheless, to the best of our knowledge, this is the first time in which the successful discrimination of displaced objects was associated with high but not low spatial displacements in rats (but see also Reichelt et al., 2021).

We then evaluated LFP activity in the dorsal hippocampus by comparing among behavioral conditions of successfull discrimination and no explicit behavioral expression of object discrimination - observed in the HD and LD test conditions, respectively. Our electrophysiological results associated increases in theta band power in the right DG to the effective discrimination of objects in conditions of pronounced spatial changes. In contrast, no similar changes in theta band power

were observed contralaterally in the left DG nor in CA3 or CA1 areas of both hemispheres. These results are in line with previous studies on hippocampal lesions, which suggest a pivotal role of the DG in the detection of spatial displacements of objects (Gilbert et al., 2001; Hunsaker and Kesner, 2008). Furthermore, other studies reported that the optogenetic silencing and pharmacological inactivation of the DG also impairs the discrimination of displaced objects in recognition memory tasks (Barbosa et al., 2012; Fernández-Ruiz et al., 2021) and during the discrimination of aversive stimuli in a spatial memory task (van Dijk and Fenton, 2018).

Our results of cross-frequency coupling also highlighted the role of the DG in the processing of recognition memory, which revealed a stronger phase-amplitude modulation between theta and fast gamma oscillations in the left DG during object exploration in HD than LD test conditions. These results paralel those of Tort et al. (2009) that found theta-slow gamma phase-amplitude modulation in CA3 during an odor-place discrimination task. The authors also found that the levels of theta-slow gamma modulation were positively correlated with memory performance. Others have associated theta-slow gamma modulation in CA1 with the successful encoding of object identity (Trimper et al., 2014). On the other hand, Fernández-Ruiz et al. (2021) reported that MEC-DG projections sustain theta-fast gamma coupling during an object-place recognition memory task, which was affected by the optogenetic perturbation of MEC. Taken together, these findings suggest that the dynamic modulation of gamma amplitude by the phases of theta oscillations throughout the hippocampus-entorhinal axis may support recognition memory. Future studies could test whether the optogenetic or chemogenetic disruption of theta oscillations or theta-gamma phase-amplitude coupling specifically during object exploration impairs performance in recognition memory tasks.

Since recognition memory tasks allow the analysis of object-associated brain activity under very similar behavior conditions, we compared theta oscillations during the exploration of stationary and displaced objects in conditions of explicit discrimination. We observed that the left CA3 expressed stronger theta power during the exploration of displaced objects at HD test condition, suggesting an involvement of CA3 theta oscillations in the detection of a new position of the familiar object. Using a NOL recognition memory task, Zheng et al. (2016) found no changes in CA3 theta power when directly comparing displaced and stationary objects. However, it may be due to the fact that in their task animals did not explicitly discriminate between object conditions in the probe session. As far as we know, no other study reported changes in CA3 theta power due to exploration of displaced and stationary objects.

In parallel to analyzing theta oscillations, Zheng et al. (2016) reported that CA1 expressed increased fast gamma band power when rats explored a new object in a new place, and suggested that fast gamma oscillations may encode new associations between place and object identity. Trimper et al. (2017) also found that slow gamma band power and coherence among DG and CA3 were associated with performance in a novel object and object-location memory task. In opposition to that, here we found no significant changes in gamma band power between high and low displacement test conditions, nor statistical changes between stationary and displaced objects. Instead, we have found a positive relationship between gamma oscillations and the discrimination index when explicit recognition memory was detected (HD test condition). In addition, the discrimination index was positively correlated with the power of both CA1 slow and fast gamma bands during the exploration of objects. Moreover, slow gamma band power was particularly associated with the exploration of stationary objects (memory retrieval). On the other hand, fast gamma band power was associated with the exploration of displaced objects (memory encoding). These results corroborate previous findings showing that slow gamma oscillations may route information from CA3 to CA1 supporting memory retrieval, while fast gamma allows direct communication between the medial entorhinal cortex and CA1, supporting memory encoding (Colgin et al., 2009; Colgin, 2016).

Our results revealed an apparent asymmetry between hippocampal hemispheres, since we found significant differences in theta band power between HD and LD conditions only in the right hemisphere and significant differences in theta-fast gamma phase-amplitude coupling only in the left hemisphere. Although some studies investigated hippocampus asymmetry (Shipton et al., 2014; Song et al., 2020; Guan et al., 2021), it is still unclear how lateralized functions could affect memory processes. It has been suggested that both left and right CA3 are involved in short-term memory, while left CA3 is essential on a long-term spatial memory task (Shipton et al., 2014). However, Song et al. (2020) found an involvement of left CA3 in a spatial working memory task. To the best of our knowledge, no studies have addressed the role of hippocampal lateralization in object recognition tasks, so at this point it is precocious to conclude whether interhemispheric asymmetry has functional importance. Future studies are needed to answer this issue.

Overall, we believe that our results are consistent with the notion that the processing of mnemonic information is supported by theta and gamma oscillatory activity in the rat hippocampus (Tort et al., 2009; Belchior et al., 2014; Colgin, 2016; Fernández-Ruiz et al., 2021). Theta and gamma oscillations are thought to foster memory encoding and retrieval providing temporal windows for effective neuronal communication and spike-timing neuronal plasticity in hippocampal circuits and

associated areas (Markram et al., 1997; Buzsáki, 2002; Fries, 2005). Therefore, our results highlight the function of different hippocampal areas on the discrimination of displaced and stationary objects, in which theta and gamma rhythms may play a critical role in the detection of spatial changes in recognition memory tasks.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This animal study was reviewed and approved by Ethics Committee on the Use of Animals (CEUA/UFRN, permit n° 52/2016).

Author contributions

FB, BL-S, and HB designed the study. LN, AF, IP, NS, AM, GN, and EG collected the data. LN, AA, and HB analyzed the data. FB, BL-S, AT, and HB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.970083/full#supplementary-material

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Endocytosis is required for consolidation of pattern-separated memories in the perirhinal cortex

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Introduction: The ability to separate similar experiences into differentiated representations is proposed to be based on a computational process called pattern separation, and it is one of the key characteristics of episodic memory. Although pattern separation has been mainly studied in the dentate gyrus of the hippocampus, this cognitive function if thought to take place also in other regions of the brain. The perirhinal cortex is important for the acquisition and storage of object memories, and in particular for object memory differentiation. The present study was devoted to investigating the importance of the cellular mechanism of endocytosis for object memory differentiation in the perirhinal cortex and its association with brain-derived neurotrophic factor, which was previously shown to be critical for the pattern separation mechanism in this structure.

Methods: We used a modified version of the object recognition memory task and intracerebral delivery of a peptide (Tat-P4) into the perirhinal cortex to block endocytosis.

Results: We found that endocytosis is necessary for pattern separation in the perirhinal cortex. We also provide evidence from a molecular disconnection experiment that BDNF and endocytosis-related mechanisms interact for memory discrimination in both male and female rats.

Discussion: Our experiments suggest that BDNF and endocytosis are essential for consolidation of separate object memories and a part of a time-restricted, protein synthesis-dependent mechanism of memory stabilization in Prh during storage of object representations.

KEYWORDS

pattern separation, object recognition, perirhinal cortex, brain derived neurotrophic factor (BDNF), endocytosis

1. Introduction

Consolidation of similar experiences as of distinct representations is a key factor for an accurate retrieval of episodic memories (Dickerson and Eichenbaum, 2010). This ability to separate similar memories into unique representations is thought to rely on pattern separation, a process of orthogonalization that has been postulated using computational

models (Marr, 1971; Treves and Rolls, 1994; McClelland et al., 1995; Rolls and Kesner, 2006). There is electrophysiological evidence that the dentate gyrus (DG) and the perirhinal cortex (Prh) are critical regions in the control of this phenomenon (Leutgeb et al., 2007; Neunuebel and Knierim, 2014; Ahn and Lee, 2017). Since episodic memory involves the recollection of unique events, separation of similar experiences is proposed to be a key component for the storage of non-confusable representations of similar experiences, especially in the hippocampus (Ranganath, 2010). Nevertheless, it has been pointed out that Prh could be an important structure involved in the consolidation of object recognition memory and where pattern separation could also occur (Zhu et al., 1995; Winters et al., 2004; Bartko et al., 2007; Miranda et al., 2017, 2020; Miranda and Bekinschtein, 2018).

Endocytosis is a fundamental process for neuronal function controlling the recycle of presynaptic vesicles, as well as the trafficking of plasma membrane receptors, ion channels, and transporters (Parton and Dotti, 1993; Haucke and Klingauf, 2007; Rosendale et al., 2017). Impairments in the endocytic pathway have been associated with the pathophysiology of certain neurological diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease (Parton and Dotti, 1993). Several studies have shown the critical role of receptor endocytosis during consolidation in different memory tasks (Abe et al., 2004; Winters and Bussey, 2005a; Dalton et al., 2008; Wang et al., 2017; Awasthi et al., 2019). Some of these receptors are critical for memory processes. Blockade of either NMDA or AMPA receptor using a broad-spectrum glutamate receptor antagonist within the anteromedial portion of Prh is sufficient to disrupt object recognition memory in macaques (Malkova et al., 2015). Interestingly, blocking the endocytosis of AMPA receptors in rat Prh prior to the retrieval phase with an interference peptide disrupted object recognition memory (Cazakoff and Howland, 2011). Nevertheless, how endocytosis interacts with other plasticity molecules to support memory, has not been studied.

Regarding the molecular mechanisms underlying memory storage, BDNF is a pivotal neurotrophin for learning and memory, including object recognition memory (Bekinschtein et al., 2014; Miranda et al., 2017). Previous studies showed BDNF mediates molecular mechanisms that are essential for the consolidation of similar and dissimilar spatial and object memories in the DG (Bekinschtein et al., 2013) and the Prh (Miranda et al., 2017, 2020). BDNF knockdown impairs long-term memory consolidation in the Prh (Seoane et al., 2011). Moreover, our group has shown that rats separate memories of ambiguous information engaging in a BDNF-associated process specifically in the Prh (Miranda et al., 2017, 2020). BDNF has a strong interaction with different types of receptors, such as glutamate receptors (AMPAr and NMDAr) and GABA receptors (GABAr) (Carvalho et al., 2008; Kealy and Commins, 2009; Lu et al., 2015; Saffarpour et al., 2017; Miranda and Bekinschtein, 2018). Phosphorylation potentiates NMDA currents in hippocampus, and it has been proposed that BDNF phosphorylation modulates NMDA receptors, enhancing synaptic transmission and playing a role in long term potentiation (Suen et al., 1997; Lin et al., 1998). In some diseases in which pattern separation is compromised, like schizophrenia, there was a clear dysregulation of AMPAr levels and BDNF signaling (Kennedy et al., 2003; Nawa and Takei, 2006; Watanabe et al., 2010; Zeppillo et al., 2020), although the functional consequence of these in this specific pathology is not yet clear. It has also been established that BDNF activates, through TrkB, a process that leads to a rapid decrease in the GABA-A receptor in the postsynaptic membrane, modulating GABA receptors trafficking (Brünig et al., 2001; Cheng and Yeh, 2003).

This study focused on the role of endocytosis in Prh and how it interacts with BDNF during consolidation of similar object memories. Since activation of the BDNF-TrkB pathway can lead to receptor endocytosis and modify synaptic plasticity, we wondered if endocytosis could be a potential molecular mechanism involved in mnemonic differentiation of objects in the Prh. Thus, this study explores a potential BDNF-dependent intracellular mechanism for discrimination of similar, but not dissimilar objects. This set of results advances further in the understanding of the molecular mechanisms of memory storage in the Prh we have been studying for many years (Miranda et al., 2017, 2020). We used a dynamin function-blocking peptide (Tat-P4) to block Prh endocytosis and found that endocytosis is necessary for consolidation of similar, but not dissimilar object memories. In addition, we provide evidence that BDNF could be interacting with pathways of endocytosis to exert its effects.

2. Materials and methods

2.1. Subjects

The subjects were 83 Long-Evans rats from our breeding colony, of which 52 were female and 31 males, that conformed mixed groups in some experiments (**Figures 1**, **2**, **3B, C**). The subjects weighed 200–300 g at the start of testing. The rats were housed on a reversed 12-h light/12-h dark cycle (lights on 19:00–07:00), in groups of two or four. All behavioral testing was conducted during the dark phase of the cycle. Rats were food deprived to 85–90% of their free feeding weight to increase spontaneous exploration, except during recovery from surgery, where food was available *ad libitum*. The water remained available *ad libitum* throughout the study. All experimentation was conducted in accordance with the Institutional Animal Care and Use Committee of the Favaloro University.

2.2. Surgery and cannulation

All rats that were used for pharmacological infusions were implanted bilaterally in Prh with 22-gauge indwelling guide cannulas. Subjects were anesthetized with ketamine (Holliday, 74 mg/kg, i.p.) and xylazine (Konig, 7.4 mg/kg, i.p.) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) with the incisor bar set at −3.2 mm. Guide cannulas were implanted according to the following coordinates, measured relative to the skull at bregma (Paxinos and Watson, 1998) anteroposterior −5.5 mm, lateral ± 6.6 mm, dorsoventral −7.1 mm. The cannulas were secured to the skull using dental acrylic. Obturators, cut to sit flush with the tip of the guide cannulas and with an outer diameter of 0.36 mm, were inserted into the guides and remained there until the first infusions. At the completion of each surgery, an antibiotic was applied for 3 days (Enrofloxacin; 0.27 mg kg-1, Vetanco, Arg). Animals were given approximately 7 days to recover before behavioral testing and drug infusions.

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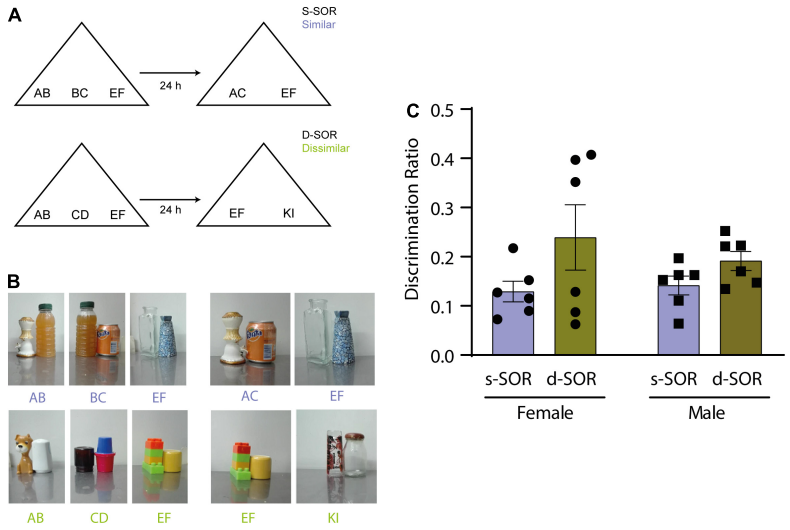

FIGURE 1
(A) Diagram of the spontaneous object recognition task (SOR) in the similar version (s-SOR) (top) and the dissimilar version (d-SOR) (up). **(B)** Examples of a set of objects used in each condition (bottom). **(C)** Discrimination ratios during the choice phase 24 h after sample phase, in the s-SOR and d-SOR condition in female and male rats. Repeated-measures two-way ANOVA; $F = 3.741$, pcondition = 0.0819, $F = 0.2857$, psex = 0.6047, $F = 0.5327$, pinteraction = 0.4822.

2.3. Infusion procedure

A Tat-conjugated peptide, designed to block the binding of dynamin to amphiphysin and thus prevent endocytosis (Gout et al., 1993; Lissin et al., 1998; Kittler et al., 2000; Lin et al., 2011), was infused in order to block endocytosis. Depending on the experiment, rats received bilateral infusions of Tat P4 peptide and Tat Scrambled control peptide (S) (60μg/μl/0.5 μl side; Cambridge, UK), human recombinant BDNF or saline (0.5 μg/μl/0.5 μl side), ANA-12 or saline at different times during the behavioral task. The injection volume was always 0.5 μl/side. Sequences are as follows: amino acid sequence for the dynamin inhibitory peptide (P4) is QVPSRPNRAP, and for the Scrambled control peptide (S) is QPPASNPRVR.

Bilateral infusions were conducted simultaneously using two 5-μl Hamilton syringes that were connected to the infusion cannulas by propylene tubing. Syringes were driven by a Harvard Apparatus precision syringe pump, which delivered 0.5 μl to each hemisphere over 1 min. The infusion cannulas were left in place for an additional minute to allow for diffusion. At least 3 days were allowed for washout between repeated infusions.

2.4. Apparatus

For the behavioral procedures, an open triangular acrylic field was used as an arena, each wall 60 cm long by 60 cm high. The walls of the triangular open field were higher to minimize the visual access to the distal cues in the room. The arena was located in the middle of a room with dim lighting, and the floor was always covered with wood shavings. A video camera was positioned on the arena in order to record both the sample session and the evaluation session for later analysis. The objects to be used were created by attaching together two small objects, depending on the condition to be studied, similar or dissimilar. Different objects were used for our within-subject design, all of them made of different materials, such as metal, glass or plastic. All of the object were approximately between 8 and 15 centimeters tall, and 4 to 7 centimeters width. All objects were adhered to the open field floor with reusable adhesive putty and cleaned with 50% ethanol solution between sessions, both sample and choice phases. For the task, the three composite objects were aligned closely to one of the arena walls and the position of each object was counterbalanced.

2.5. Behavioral procedures

For the Spontaneous Object Recognition (SOR) task (Figure 1A), each rat was handled for 3 days and then habituated to the arena for 5 min per day for 3 days before exposure to the objects. After habituation, the rats were exposed during a 5 min duration sample phase to three objects made of two features depending on the condition. For the similar condition, two of the objects shared one feature (AB and BC) and the third object was made of two other different features (EF). For the dissimilar condition, all three objects were made of different features (AB, CD, and EF). Twenty-four hours after sample phase, we conduct a choice phase, of 3 min duration, in which the animals were exposed to two objects, a novel one and a familiar one, and depending on the condition evaluated, the objects varied in composition. For the similar condition, the novel object was made of the two non-shared features of the objects presented in the sample phase (AC), and the familiar object was a copy of the third object (EF). For the dissimilar condition, the novel object was made of two novel features (KI) and the familiar object was a copy of the object presented during the sample phase (AB, CD, and EF).

For the extra-similar condition, the process was the same as the similar condition, differing only in the objects used. During

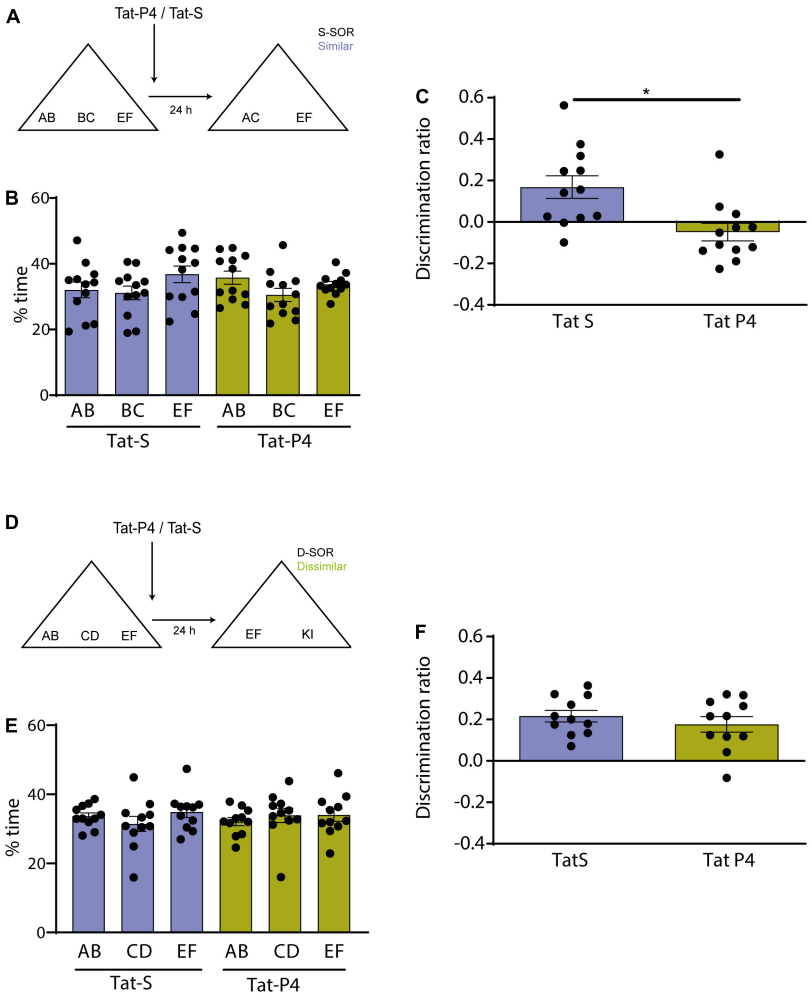

FIGURE 2
(A) Schematic illustration of the similar-spontaneous object recognition task (s-SOR) task indicating when Tat-P4/Tat-S was infused. **(B)** Percentage of time spent exploring each of the objects in the sample phase in the s-SOR. Repeated-measures two-way ANOVA (%time); $F = 1.000$, pdrug = 0.3388, $F = 2.045$, pobject = 0.1533, $F = 0.7906$, pinteraction = 0.4660. **(C)** Effect of Tat-P4 or Tat-S injections on the discrimination ratios for the s-SOR version of the task. Paired t-test ($t = 2.899$), $p = 0.0145$, $n = 12$. **(D)** Schematic illustration of the dissimilar (d-SOR) task indicating when Tat-P4/Tat-S was infused. **(E)** Percentage of time spent exploring each of the objects in the sample phase in the d-SOR. Repeated-measures two-way ANOVA (%time); $F = 0.1020$, pdrug = 0.7560, $F = 0.4530$, pobject = 0.6421, $F = 0.5049$, pinteraction = 0.6111. **(F)** Effect of Tat-P4 or Tat-S injections on the discrimination ratios for the d-SOR version of the task. Paired t-test ($t = 0.8728$), $p = 0.4033$, $n = 11$. *$p < 0.05$.

sample phase, animals were exposed during 5 min to three different objects, and two of those shared one feature (ABB and BBC), while the third object was made of different features (EFG). During the choice phase, 24 h later, the animals were exposed to a novel object was made of a novel combination of familiar features (ABC), and the familiar object was a copy of the third object presented in the sample phase (EFG) (Figure 3D). Exploration was recorded and later scored manually for both the sample and choice phases. For all experiments, exploration of a particular object was defined as the rat having its nose directed at the object at 2 cm or less or touching the object with its nose. Rearing with the head oriented upward did not count as exploration. Climbing over or sitting on the object was not included.

In every trial, objects were pseudorandomly assigned to a different location in the arena to avoid a bias for locations within the arena.

2.6. Statistical analysis

For all the experiments, the results were expressed as a discrimination ratio calculated as the time exploring the novel object minus the time exploring the familiar object divided by the total exploration time [(tnovel-tfamiliar)/(ttotal)].

For the sample phases, the percentage of time spent exploring each object was compared using a repeated-measures two-way ANOVA or one-way ANOVA. For the choice phases, we performed one-sample t-test for every discrimination ratio to analyze whether control animals learned the task, verifying that the ratio was different from zero. Discrimination ratios were compared within subject using a paired t-test, one way ANOVA or two-way ANOVA.

Some experiment has female and male animals, and the statistical analyses was made with pooled data, except for the first experiment (Figure 1). The experiments in which there are animals

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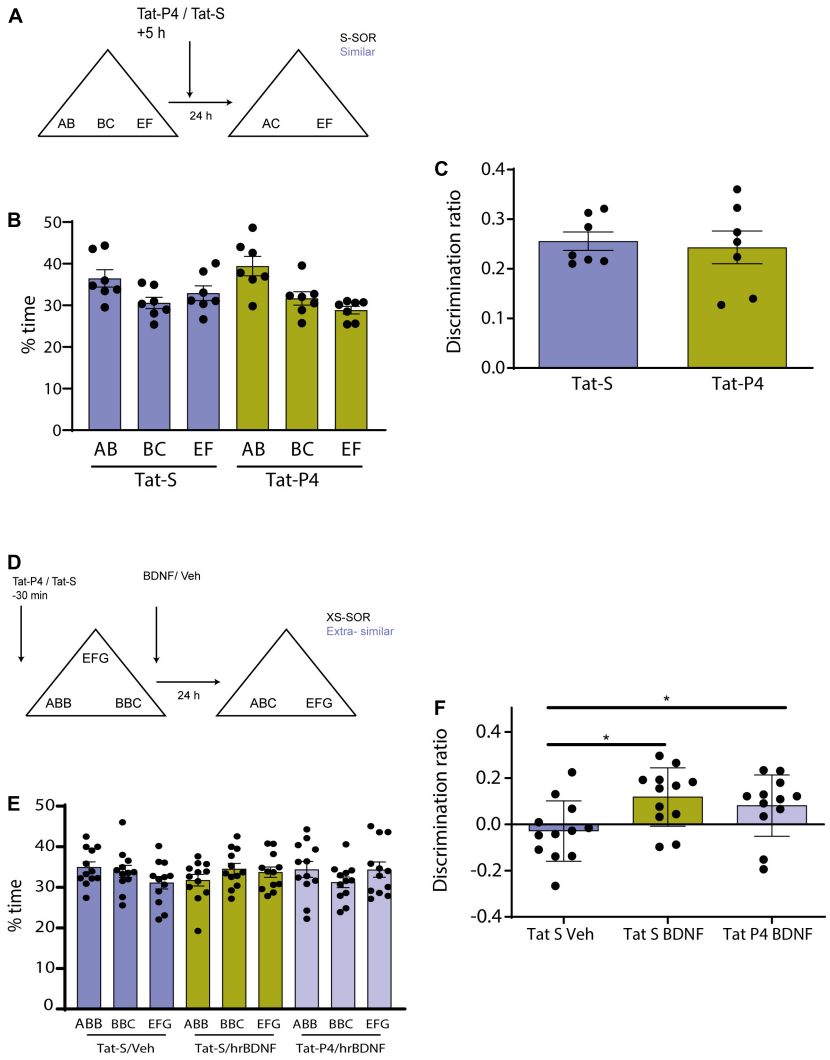

FIGURE 3
(A) Schematic illustration of the similar-spontaneous object recognition task (s-SOR) task indicating infusion of Tat-P4/Tat-S 5 hs after the sample phase. **(B)** Percentage of time spent exploring each of the objects in the sample phase. Repeated-measures two-way ANOVA (%time) $F = 2.400$, pdrug = 0.1723, $F = 6.509$, pobject = 0.0122, $F = 01.608$, pinteraction = 0.2407. **(C)** No memory impairment found in animals between conditions [Tat-S vs. Tat-P4 paired t-test ($t = 0.2985$), $p = 0.7754$, $n = 7$]. Both animal groups learned the task [One-sample t-test (Tat-S, $t = 13.88$), $p < 0.0001$; one- sample t-test (Tat-P4, $t = 7.377$), $p = 0.0003$]. **(D)** Schematic illustration of the extra similar condition of the task (xs-SOR) indicating the time points at which Tat-P4 or Tat-S and hrBDNF or vehicle were infused 30 min before sample phase and immediately after sample phase, respectively. **(E)** Percentage of time spent exploring each of the objects in the sample phase. Repeated-measures two-way ANOVA (%time), $F = 1.000$, pdrug = 0.1748, $F = 0.1089$, pobject = 0.8668, $F = 1.191$, pinteraction = 0.3282. **(F)** Repeated measures one-way ANOVA (%time), $F = 3.838$, pdrug = 0.0430, $F = 0.7578$, pindividual = 0.6757. *$p < 0.05$.

of both sexes, the number of animals of each sex is detailed in the results.

3. Results

3.1. Female rats, as well as male rats, can spontaneously store and disambiguate the representations of similar and dissimilar objects

It has already been shown that object exploration and preference is driven by novelty in the modified version of the SOR task in male rats (Miranda et al., 2017). This task includes a similar and a dissimilar condition in which the load on pattern separation is different (Figure 1A; see M and M). The first goal of this work was to test the performance of female rats in the same SOR task version and compare the result with male rats (Figure 1).

Six male Long Evans and 6 female Long Evans were used for this experiment, all animals underwent both the similar (s-SOR) and dissimilar (d-SOR) conditions. We did not find a difference in the percentage of time the animals spend exploring the objects during the sample phase {repeated measures one way ANOVA: females s-SOR [F (1.588, 7.942) = 1.829, $p = 0.2211$]}; males s-SOR [F (1.147, 5.734) = 3.364, $p = 0.1171$]; females d-SOR [F (1.457, 7.287) = 3.202, $p = 0.1081$]; males d-SOR [F (1.438, 7.190) = 2.240, $p = 0.1779$)]. There was no significant interaction

between the objects by condition and sex (repeated measures two-way ANOVA: psex = 0.6109, pcondition + object = 0.0745, pinteraction = 0.2793).

Also, we did not find any differences in total exploration times (female s-SOR = 39.78 ± 5.09, male s-SOR = 39.63 ± 5.21, female d-SOR = 30.08 ± 3.94, male d-SOR = 36.88 ± 1.92) comparing sexes in the same condition (paired *t*-test: female versus male similar, $p = 0.9840$; female versus male dissimilar, $p = 0.1518$).

The choice phase was conducted 24 h after the sample phase for both conditions and memory was evaluated by comparing the amount of time spent exploring a novel object and familiar object. This comparison was expressed in the discrimination ratio. There were no significant differences between the discrimination ratio for both sexes in the similar and the dissimilar condition (pconditions = 0.0819, psex = 0.6047, pinteraction = 0.4822) (**Figure 1B**, **Supplementary Table 1**). Also, we did not find any differences in total exploration times comparing sexes in the same condition (paired *t*-test: female versus male similar, $p = 0.9840$; female versus male dissimilar, $p = 0.1518$) (**Table 1**). One-sample *t*-tests against a value of zero indicated that both sexes were able to learn the task in both conditions [One-sample *t*-test (s-SOR females, $t = 6.146$), $p = 0.0017$; one- sample *t*-test (s-SOR males, $t = 6.740$), $p = 0.0011$; d-SOR females, $t = 3.277$), $p = 0.0220$; one-sample *t*-test (d-SOR males, $t = 5.128$, $p = 0.0037$)].

These results indicate that intact female rats were able to spontaneously disambiguate the representations of two similar object seen 24 h before, and that there is no difference between sexes for performance in this specific task.

3.2. Endocytosis in the Prh is required for consolidation of similar, but not for dissimilar object memory representations

We then proceeded to study the role of the endocytosis in the formation of differentiated representations in the Prh in male and female rats. If mechanisms of receptor internalization are specifically required in the Prh for memory differentiation, then blocking internalization should alter the similar version of the SOR task, without affecting the dissimilar version. To test this hypothesis, we blocked the putative receptor internalization immediately after the sample phase. We used a Tat-conjugated peptide (Tat-P4) and a scrambled control peptide (Tat-S) design to prevent endocytosis by blocking the binding of dynamin (Gout et al., 1993; Lissin et al., 1998; Lin et al., 2011). Tat-P4 or Tat-S was injected in Prh immediately after the sample phase, and the memory of animals was test 24 h later in both conditions, similar ($n = 12$, 6 male and 6 female) and dissimilar ($n = 11$, 5 male and 6 female) (**Figures 2A, D**). Animals from both sexes were pooled and analyzed altogether, and all animals underwent the experimental (Tat-P4) and the control (Tat-S) conditions. A discrimination index above zero indicates a significant discrimination and a reasonable memory retention. One-sample *t*-tests against a value of zero indicated that Tat-S injected animals were able to learn both the s-SOR and the d-SOR [One-sample *t*-test (d-SOR Tat-S, $t = 7.735$), $p < 0.001$; one- sample *t*-test (s-SOR Tat-S, $t = 3.071$), $p = 0.0106$] (**Figures 2C, F**), whereas Tat-P4 injected animals only learned the d-SOR version [One-sample *t*-test (d-SOR Tat-P4, $t = 4.655$), $p = 0.0009$; one- sample *t*-test (s-SOR Tat-P4, $t = 1.142$), $p = 0.2776$)] (**Figures 2C, F**). We found a significant difference between Tat-S and Tat-P4 injected animals in the choice phase in the s-SOR [Tat-S vs. Tat-P4 paired *t*-test ($t = 2.899$), $p = 0.0145$, $n = 12$] (**Figure 2C**). There were no differences in total exploration times between groups (see **Table 1**). These results indicate that endocytosis is important to spontaneously disambiguate the memory representations of two similar objects.

3.3. Endocytosis is required in a time-restricted windows for consolidation of similar object memory representations

Memory consolidation is a process that occurs during a restricted time window (McGaugh, 2000; Winters and Bussey, 2005b). To test whether Tat-P4 interfered with memory during a restricted delay after the sample phase, Tat-P4 or Tat-S was injected into the Prh 5 h after the sample phase, and rats were tested 24 h later, all animals underwent both drug conditions ($n = 7$, 2 males, 5 female) (**Figure 3A**). Since in the previous experiment we found an effect only in the similar condition, we decided to test this time window in this specific condition. Injection of the TatP4 did not change total exploration times compared with Tat-S (see **Table 1**). We did not observe any memory impairments in the s-SOR when Tat-P4 was injected 5 h after sampling the objects [One-sample *t*-test (Tat-S, $t = 13.88$), $p < 0.0001$; one- sample *t*-test (Tat-P4, $t = 7.377$), $p = 0.0003$], indicating that Tat-P4 injected animals were able to learn the similar condition as successfully as Tat-S-injected animals [Tat-S vs. Tat-P4 paired *t*-test ($t = 0.2985$), $p = 0.7754$, $n = 7$] (**Figure 3C**). In sum, this result indicates that extending the time interval between sample phase and Tat-P4 infusion reduces the disrupting effect of the peptide on the choice phase.

3.4. Is BDNF acting through endocytosis to promote differentiation discrimination?

Brain derived neurotrophic factor enhances memory consolidation in several tasks if injected exogenously (Alonso et al., 2002; Peters et al., 2010; Bekinschtein et al., 2013). We wondered if the enhancing effect found in previous studies could be prevented if we blocked the internalization of receptors. We have previously shown that BDNF in Prh is critical for this task. In particular, injection of exogenous BDNF into Prh enhanced discrimination of similar object memories (Miranda et al., 2017). To be able to see any memory improvement induced by BDNF in pattern separation, we used a slightly different version of the SOR task, the extra-similar SOR (xs-SOR) (Miranda et al., 2017) in which we make discrimination more difficult by bringing the performance of the control animals down. The key modification to the task is making the objects more similar during the sample phase. In this particular experiment, we used three groups of rats, a

group injected in Prh with Tat-S and saline 15 min prior to sample phase, a second group injected with Tat-S 15 min prior and human recombinant BDNF (hrBDNF) immediately after the sample phase, and a third group injected with Tat-P4 15 min prior and hrBDNF immediately after sample phase. All animals were exposed to the three treatments ($n = 12$, all males) (Figure 3D).

There were no differences in exploration of the three objects during the sample phase (repeated-measures two-way ANOVA, xs-SOR: $F = 1.000$, pdrug $= 0.1748$; $F = 0.1089$ pobjects $= 0.8668$, $F = 1.191$, pinteraction $= 0.3282$) (Figure 3E). A one-way between-subjects ANOVA was conducted to compare the effect of the treatments in the choice phase. There was a significant difference between treatments at the $p < 0.05$ level [F (Rolls and Kesner, 2006; Miranda et al., 2017) $= 3.838$, $p = 0.043$]. *Post hoc* comparison using Tukey's multiple comparison test indicated that the mean score for the Tat-S/Veh condition ($M = -0.02$, SEM $= 0.037$) was significantly different than the Tat-S/BDNF condition ($M = 0.11$, SEM $= 0.036$) and different than the Tat-P4/BDNF condition ($M = 0.08$, SEM $= 0.038$). However, the Tat-S/BDNF condition did not significantly differ from the Tat-P4/BDNF condition (Figure 3F). This experiment was inconclusive, as it appears that BDNF was not able to improve discrimination in Tat-P4-injected animals, but the discrimination index did not differ from that of the Tat-S/BDNF group.

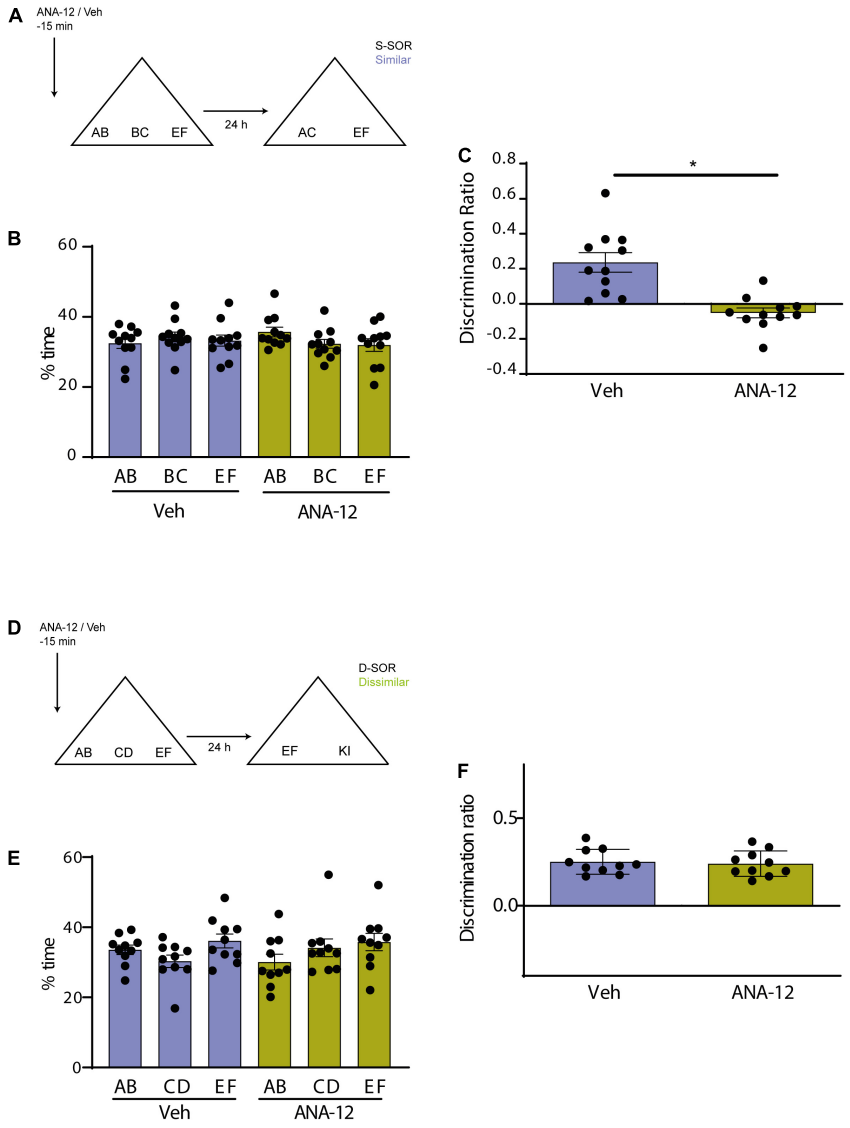

FIGURE 4
(A) Schematic illustration of the similar-spontaneous object recognition task (s-SOR) task indicating when ANA-12/Veh was infused 15 min before the sample phase. **(B)** Percentage of time spent exploring each of the objects in the sample phase. Repeated-measures two-way ANOVA (%time) $F = 1.000$, pdrug $= 0.3409$, $F = 0.3029$, pobject $= 0.7420$, $F = 1.342$, pinteraction $= 0.2839$. **(C)** Effect of ANA-12 or Veh injections on the discrimination ratios for the s-SOR version of the task. Paired t-test, $p = 0.0005$, $t = 4.995$, $n = 11$. **(D)** Schematic illustration of the d-SOR task indicating when ANA-12/Veh was infused 15 min before the sample phase. **(E)** Percentage of time spent exploring each of the objects in the sample phase. Repeated-measures two-way ANOVA (%time) $F = 1.976$, pdrug $= 0.1934$, $F = 2.066$, pobject $= 0.1557$, $F = 0.8339$, pinteraction $= 0.4504$. **(F)** Effect of ANA-12 or Veh injections on the discrimination ratios for the d-SOR version of the task. Paired t-test, $p = 0.0005$, $t = 4.995$, $n = 10$. *$p < 0.05$.

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TABLE 1 Total exploration times during the choice session of the SOR task.

Figure	*P*-value	T total
1C	0.9840	
s-SOR female		39.78 ± 5.09
s-SOR male		39.63 ± 5.21
	0.1518	
d-SOR female		30.08 ± 3.94
d-SOR male		36.88 ± 1.92
2C	0.3567	
s-SOR Tat-s		35.66 ± 6.05
s-SOR Tat-P4		29.52 ± 4.32
2F	0.1128	
d-SOR Tat-s		45.42 ± 3.84
d-SOR Tat-P4		36.77 ± 3.83
3C	0.0792	
s-SOR Tat-S		32.17 ± 2.83
s-SOR Tat-P4		27.04 ± 2.24
3F		
Tat-S/Vehicle		57.57 ± 4.97
Tat-S/hrBDNF		53.36 ± 5.06
Tat-P4/hrBDNF		49.58 ± 7.34
4C	0.8451	
Vehícle		25.68 ± 1.25
ANA-12		26.15 ± 2.06
4F	0.8111	
Vehicle		38.44 ± 3.70
ANA-12		37.57 ± 1.84
5C	0.6272	
Unilateral		46.84 ± 3.93
Contralateral		44.15 ± 1.88

Results are expressed as mean ± SEM in seconds. *P*-values are for the comparison between total exploration times during the choice session for each experimental group depicted in the same row. Paired *t*-test was used for these comparisons, except in the case of Figure 3F, for which one-way ANOVA was used.

TABLE 2 Total exploration times during the sample session of the spontaneous object recognition (SOR) task.

Figure	*P*-value	T total
2B	0.3321	
s-SOR Tat-s		81.70 ± 8.71
s-SOR Tat-P4		90.58 ± 9.33
2E	0.9826	
d-SOR Tat-s		75.06 ± 6.43
d-SOR Tat-P4		74.76 ± 10.61
3B	0.3453	
s-SOR Tat-S		82.74 ± 4.63
s-SOR Tat-P4		74.30 ± 5.65
3E		
Tat-S/Vehicle		125.9 ± 10.55
Tat-S/hrBDNF		126.4 ± 11.7
Tat-P4/hrBDNF		117.7 ± 14.6
4B	0.4431	
Vehícle		61.27 ± 6.76
ANA-12		54.15 ± 4.53
4E	0.8804	
Vehicle		64.45 ± 3.61
ANA-12		63.52 ± 4.68
5B	0.5426	
Unilateral		91.72 ± 10.18
Contralateral		85.97 ± 6.09

Results are expressed as mean ± SEM in seconds.

3.5. Interaction between BDNF–TrkB signaling and endocytosis in pattern separation

We wanted to use a different strategy to evaluate the possible interaction between BDNF and endocytosis by blocking both the BDNF receptor TrkB and endocytosis in a molecular disconnection experiment (Miranda et al., 2017). We first tested the effect of Prh injection of ANA-12, a selective non-competitive antagonist of TrkB, BDNF receptor (Cazorla et al., 2011). We injected all animals with ANA-12 or saline in Prh 15 min prior to the sample phase and evaluated memory 24 h after training in both similar and dissimilar version (**Figures 4A, D**). There were no differences in total exploration times in neither the similar (n = 11, all female) nor the dissimilar (n = 10, all female) version of the task (paired t-test, similar, p = 0.6742; dissimilar, p = 0.8804) (**Table 2**). We did not find any interactions between drugs or objects (repeated-measures two-way ANOVA, d-SOR: pdrug = 0.1934, pobjects = 0.1557, pinteraction = 0.4504; s-SOR: pdrug = 0.3409, pobjects = 0.7420, pinteraction = 0.2839) (**Figures 4B, E**). We observed a memory impairment in the s-SOR version of the task (paired t-test, p = 0.0005, t = 4.995) (**Figure 4C**), but not in the d-SOR version (paired t-test, p = 0.7462, t = 0.3337) (**Figure 4F**), Thus, blocking TrkB only generated a deficit in the "similar" condition, disabling animal's capacity of discrimination of overlapping memories.

We next evaluated whether BDNF pathway and endocytosis interacted during consolidation of similar object memories in the SOR task. We used a protocol of molecular disconnection that we have carried out in previous studies (Miranda et al., 2017). The logic underlying this is the same that in any brain disconnection experiment that tries to determinate if, during a specific behavioral manipulation, two brain structures are connected (Gaffan et al., 1989; Ito et al., 2008). If we assume that the principal connection between two structures is in the same hemisphere (ipsilateral), the deactivation or lesion of the two regions in the same side will keep the behavior intact, but the contralateral deactivation will affect the performance. If we consider two molecules or gene expression pathways in specific given structure instead of two regions, a similar method of reasoning can be used. If two molecular pathways

interact to produce a specific behavior, blocking both pathways in that area of only one hemisphere will have no effect; but if one pathway is blocked in one hemisphere and the second pathway in the other, we would see a deficit. Thus, we evaluated if BDNF and endocytosis interacted in Prh during consolidation of similar memories by blocking both pathways in the same hemisphere or blocking BDNF in one hemisphere and endocytosis in the other. We injected ANA-12/Veh or Tat-P4/Tat-S in Prh 15 min before the sample phase and evaluated memory 24 h after it ($n = 8$, all female) (**Figure 5A**). All animals underwent both treatment conditions. There were no differences in total exploration time between the two groups (paired t-test, $p = 0.5426$, $t = 0.6399$, $n = 8$), nor between objects (repeated-measures two-way ANOVA, s-SOR: pcondition = 0.7318, pobject = 0.1943, pinteraction = 0.4355) (**Figure 5B**).

We found no effect in the similar SOR task when ANA-12 and TatP4 were injected in the same hemisphere (and their corresponding vehicles were injected in the other hemisphere) (**Figure 5C**), however, when ANA-12/TatS and Veh/TatP4 where injected in different hemispheres in Prh, we found a significant impairment in the similar SOR task (**Figure 5C**) (paired t-test unilateral vs. contralateral, $p = 0.0090$, $t = 3.574$). There were no differences in total exploration times between the two groups (see **Table 1**). One sample t-test showed that the discrimination ratio from the "unilateral" group was different from zero, whereas the discrimination ratio from the "contralateral" group was not ($p_{unilateral} = 0.0210$, $t = 2.96$; $p_{contralateral} = 0.0686$, $t = 2.150$). This result suggests that BDNF and endocytosis interacts during consolidation of similar memories in Prh.

4. Discussion

The key finding of this study are: (1) blocking endocytosis in Prh impairs consolidation of similar, but not dissimilar object memories, (2) blocking BDNF TrkB receptors prevents consolidation of similar objects in Prh, (3) an interaction between endocytosis and BDNF is necessary for appropriate memory differentiation, and (4) we found no sex differences in this particular task. In accordance with previous studies that have shown the importance of the different receptors in memory consolidation (Winters and Bussey, 2005a; Banks et al., 2014; Sanchez-Mejias et al., 2020), we contribute with evidence that shows that endocytosis in general affects memory consolidation of similar representations during an object recognition task in Prh.

In this study, we hypothesize that impairing receptor trafficking interferes with the plasticity of Prh, disturbing memory consolidation. This is consistent with previous findings showing that blocking receptor trafficking impairs object recognition memory. For example, the blockade of NMDARs after the sample phase impaired both object recognition memory (Winters and Bussey, 2005a) and long- term potentiation (LTP) (Barker et al., 2006). A study using a Tat-conjugated peptide to block the endocytosis of AMPAR also impaired object recognition memory, but only when injected prior to the retrieval phase (Cazakoff and Howland, 2011), while a short-term treatment with Flumazenil, a GABAr antagonist, improved long term memory in the Novel Object Recognition task in a mouse model of Down's Syndrome, which is characterized by a cognitive deficit generated by excessive neuronal inhibitory tone (Colas et al., 2017). In this study, we bounce into endocytosis-dependent trafficking, impairing object recognition memory. Endocytosis could act to facilitate memory differentiation by different mechanisms: (1) AMPA receptor internalization could be leading to a greater malleability of plasticity mechanism or (2) GABA receptor endocytosis could decrease postsynaptic inhibition, hence facilitating synaptic plasticity. If indeed it were so, the interference in the mechanisms of internalization of receptors would lead to a non-discrimination behavior when the pattern separation load is high but not when it is low. Due to the fact that we used a general method to impair endocytosis, we cannot establish the trafficking of which particular receptors was affected.

Tat-P4 is a dynamin inhibitory peptide, it has been designed to block the binding of dynamin to amphiphysin, and thus it prevents endocytosis. Blockade of the endocytic pathway by this peptide has been more thoroughly studied *in vitro* (Zheng et al., 2008; Cosker and Segal, 2014; Moya-Alvarado et al., 2018; González-Gutiérrez et al., 2020). We did not perform a thorough time course analysis of the Tat-P4 peptide blocking action. In a study involving fear-potentiated startle and infusion of Tat-P4 in the amygdala, the authors found a strong behavioral (impaired reinstatement) and molecular effect (blockade of GABAr endocytosis) when the peptide was injected 30 min before testing (Cosker and Segal, 2014). In a study using cultured cells, a plateau on GABA-A mIPSC increase was found around 40 to 50 min after treatment with the Tat-P4 peptide (Lin et al., 2011). In our experiments, we only found an effect on behavior when Tat-P4 was injected either 15 min before the sample phase or immediately after, but not 5 h after. This is consistent with previous studies showing that the blocking action of the peptide begins within minutes (Zheng et al., 2008; Cosker and Segal, 2014; Moya-Alvarado et al., 2018; González-Gutiérrez et al., 2020).

Changes in synaptic strength are thought to support long-term memory in the brain (Kandel, 2001). It has been proposed that LTP and long-term depression (LTD) are key processes underlying memory storage in several different neural regions (Bliss and Collingridge, 1993; Martin et al., 2000; Kandel, 2001). In particular, both of these forms of synaptic plasticity have been found in Prh (Bilkey, 1996; Ziakopoulos et al., 1999; Cho et al., 2000; Massey et al., 2001), Nevertheless, object recognition memory has been strongly linked with the induction and maintenance of LTD in this particular structure [see review (Miranda and Bekinschtein, 2018)]. Both LTP and LTD involve AMPAR and NMDAR-dependent mechanisms in the Prh (Bilkey, 1996; Ziakopoulos et al., 1999). It has been shown that NMDAR-dependent LTD requires the internalization of AMPA receptors in Prh (Griffiths et al., 2008), while LTP is associated with perirhinal NMDAR (Barker et al., 2006) and was shown to also recruit GABA-dependent mechanisms in this structure (Kotak et al., 2017). While there are currently many results that point at LTD as the key mechanism of synaptic plasticity for object recognition in the Prh, it is possible that a balance between LTD and LTP is needed for the maintenance of consolidation and storage of distinguished representations of object memories (Miranda and Bekinschtein, 2018).

Brain derived neurotrophic factor is considered to be an important part of the cellular mechanism that supports the formation and maintenance of memory by promoting synaptic

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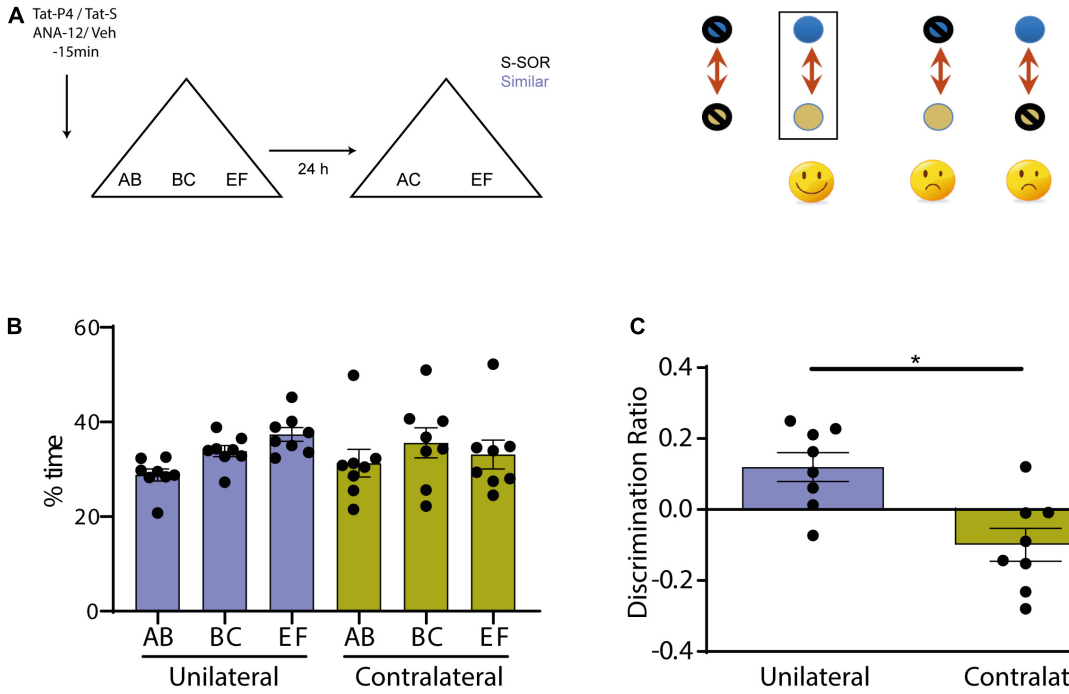

FIGURE 5
(A) Schematic illustration of the similar-spontaneous object recognition task (s-SOR) task indicating when ANA-12/TatS and Veh/TatP4 was infused (left). In a molecular disconnection experiment, there is an inactivation of two elements of a signal transduction pathway either in the same hemisphere or in both of them. While in the first case, the putative pathway that links both proteins remain intact and functional in one hemisphere, the inactivation of one element in one hemisphere and the other element in the other hemisphere prevents functionality of the putative interaction pathway in both hemispheres (right). **(B)** Percentage of time spent exploring each of the objects in the sample phase. Repeated-measures two-way ANOVA (%time), s-SOR: pcondition = 0.7318, pobject = 0.1943, pinteraction = 0.4355. **(C)** We found no effect in the similar SOR task when ANA-12 and TatP4 were injected in the same hemisphere [One-sample *t*-test (same, $t = 2.964$), $p = 0.0210$]. However, when ANA-12/TatS and Veh/TatP4 where injected in different hemispheres in Prh, we found a significant impairment in the similar SOR task [One-sample *t*-test (different, $t = 2.150$), $p = 0.0686$; paired *t*-test same vs. different, $p = 0.0090$, $t = 3.574$, $n = 8$]. *$p < 0.05$.

consolidation. Accordingly, BDNF also generates changes in spine shape, leading to the stabilization of LTP and, as a result, increased memory storage (Bramham and Messaoudi, 2005). Previous studies have also shown the importance of BDNF in object recognition memory (Seoane et al., 2011). In our studies, we found that blocking the expression BDNF in Prh using a BDNF antisense oligonucleotide impaired only the performance in the similar condition of the SOR task, showing the existence of a specific mechanisms underlying storage of unique representations of objects in Prh (Miranda et al., 2017). In this particular study, we did not block the expression of BDNF, instead we used a not competitive antagonist (ANA-12) to prevent the activation of BDNF receptor, TrkB, obtaining similar results. Inhibiting TrkB impaired memory consolidation of similar but not distinct objects. We also evaluated if BDNF and endocytosis signaling pathways are connected in Prh using a molecular disconnection experiment. The result suggests that BDNF and endocytosis interact during consolidation of overlapping memories in Prh. However, we also tested if human recombinant BDNF could enhance object recognition memory in an endocytosis-dependent manner. We predicted that the enhancing effect of BDNF would be prevented when we blocked the trafficking receptor. We found that BDNF did enhance the consolidation of extra-similar memories, but we did not find a significant effect of blocking endocytosis using Tat-P4. However, animals injected with BDNF and Tat-P4 seem to remember worse than BDNF control animals. There are a number of reasons why this experiment was not conclusive. For example, exogenous BDNF might engage a different mechanism than that of physiological BDNF in which endocytosis is partially required. In addition, the dose of Tat-P4 peptide could not have been enough to block the effect of a large exogenous BDNF dose. Our current data is not sufficient to make a conclusion form this particular experiment. Nevertheless, the robustness of the molecular disconnection result strongly suggests that there is an interaction between BDNF and endocytosis in the Prh during consolidation of similar overlapping object memories.

In conclusion, our experiments suggest that BDNF and endocytosis are essential for consolidation of separate memories and a part of a time-restricted, protein synthesis-dependent mechanism of memory stabilization in Prh during storage of object representations. These results agree with previous investigation that showed the critical importance of BDNF for this type of memories and the molecular mechanisms underlying this process (Miranda et al., 2017).

To our knowledge, the present study is the first to provide evidence regarding the role of endocytosis in the consolidation of overlapping memories in the Prh and the first to test this task in female rats. Together with previous studies, we reinforce the importance that BDNF as a plasticity molecule involved in this process across different brain regions.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

All experimentation was conducted in accordance with the Institutional Animal Care and Use Committee of the Favaloro University, Buenos Aires, Argentina.

Author contributions

DP was responsible for all the experiments and drafting the manuscript. MM contributed to the experiments. MG was responsible for critically revising and correcting the manuscript. PB was responsible for the general idea and critically revising and correcting the manuscript. NW contributed to the general idea and final revision of the manuscript. All authors read and approved the final manuscript and contributed to the conception of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnsys.2023.1043664/full#supplementary-material

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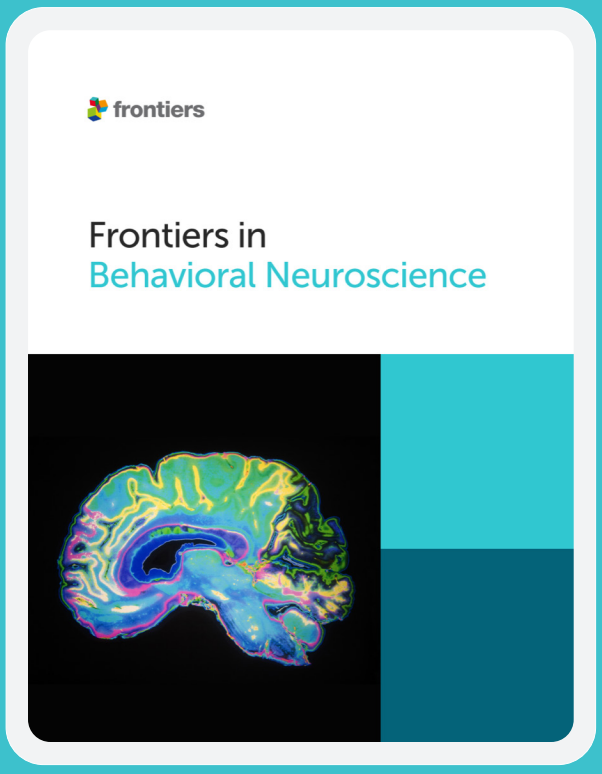

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