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Lorena Roselló-Jiménez
Universitat Jaume I
Raúl Pastor
Universitat Jaume I
Marta Miquel
Universitat Jaume I
Laura Font
laura.font@uji.es
Universitat Jaume I

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Sex-Specific Exploration Patterns and Noradrenergic Modulation in Object Recognition Memory: Role of the Perirhinal Cortex

Lorena Roselló-Jiménez, Raúl Pastor, Marta Miquel, Laura Font*

Área de Psicobiología, Universitat Jaume I, Castellón de la Plana, Spain

*Corresponding author:

Laura Font, Ph.D.

Área de Psicobiología
Facultad de Ciencias de la Salud
Universitat Jaume I
Avda. Sos Baynat s/n, 12071
Castelló de la Plana, Spain.
laura.font@uji.es
+34 964 729 843

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Author contributions

LRJ contributed to study conceptualization and design, conducted behavioral and immunochemistry experiments, performed data and statistical analyses, prepared figures, and drafted and revised the manuscript. RP contributed to study conceptualization, manuscript revision and editing. MM contributed to study conceptualization, manuscript revision and editing as well as funding acquisition. LF contributed to study conceptualization and design, manuscript preparation and revision, as well as supervision, project administration, and funding acquisition.

Data availability

The data underlying this study's findings can be provided by the corresponding author upon request.

ABSTRACT

Introduction. Object recognition memory (ORM) relies on two processes: familiarity, mediated by the perirhinal cortex (PRH), and recollection, supported by the hippocampus (HIP). These processes are recruited depending on memory strength: brief exploration forms weak traces relying on PRH familiarity, whereas extended exploration produces strong traces requiring HIP recollection. Noradrenaline (NA) modulates these mechanisms via β2-adrenergic receptors (β2-AR), influencing memory strength and consolidation. However, whether this modulation differs between sexes under conditions generating weak versus strong memory traces remains unknown. We examined how NA and β2-AR influence ORM in male and female mice under these conditions.

Methods. Swiss mice were tested in the Novel Object Recognition task using training durations of 1–10 minutes and delays of 24–48 hours. Atomoxetine (ATO) was used to increase NA levels, and ICI-118,551 (ICI) to block β2-AR. Neural activation was assessed via c-Fos immunohistochemistry in PRH and HIP.

Results. Females showed greater efficiency in object processing, requiring less exploration than males. Under weak training conditions, ATO facilitated the formation of long-term ORM in females but not in males, accompanied by increased PRH c-Fos expression without HIP involvement. β2-AR blockade impaired ORM acquisition only in females, with no significant changes in c-Fos. In males, performance depended on object dissimilarity; increased NA did not enhance ORM unless stimuli were highly distinct.

Conclusions. These findings reveal sex differences in noradrenergic modulation of ORM. In females, NA acting via β2-AR supports memory formation through PRH-dependent familiarity, emphasizing the importance of sex as a biological variable in research.

Keywords. Object Recognition Memory, Noradrenaline, β_2-adrenergic Receptors, Perirhinal Cortex, Hippocampus, Sex, Novel Object Recognition Task

INTRODUCTION

Memory is a fundamental cognitive function that enables organisms to integrate past experiences to guide future behavior, thereby promoting learning, adaptation, and survival. It is classified according to the duration over which information is retained, with short-term memory supporting transient maintenance over brief intervals, and long-term memory enabling stable storage across extended periods (Alberini 2009; Veloz Castillo et al. 2021). Within long-term memory, declarative memory constitutes a major subtype and refers to the conscious recall of facts, personal experiences, and general knowledge, providing a basis for flexible behavior in dynamic environments. Recognition memory, a form of declarative memory, involves identifying previously encountered environments and their stimuli (Lang et al. 2023). This function is essential for effective environmental interaction and behavioral regulation. Recognition memory relies on two distinct processes: recollection and familiarity. Recollection involves retrieving an experience along with its contextual details, such as spatial or temporal information, whereas familiarity refers to recognizing a stimulus without recalling its context. Traditionally, these processes have been attributed to different neural substrates. Recollection requires integration of "what" and "where" information and primarily recruits the hippocampus (HIP), whereas familiarity is mediated by the perirhinal cortex (PRH), specialized in encoding "what" information (Chao et al. 2022; Cohen & Stackman 2015; Veloz Castillo et al. 2021; Wan et al. 1996; Ward 2019). However, this dichotomy has been challenged by evidence that both PRH and HIP may be recruited depending on exploration, which determines memory trace strength (Cinalli et al. 2020; Cohen & Stackman 2015; Squire et al. 2007). Accordingly, brief exploration during encoding yields a weaker trace relying on the PRH, whereas more extensive exploration induces a stronger trace dependent on the HIP (Cinalli et al. 2020). Recognition memory is markedly impaired in memory-related dementias such as Alzheimer's disease and dementia with Lewy Bodies, but also in disorders not primarily defined by memory deficits, including Parkinson's disease (Chiaravalloti et al. 2014; Hart et al. 1985; Raposo Pereira et al. 2025). Notably, recognition-based tests can detect dementia onset, as deficits are observable up to four years before diagnosis (Raposo Pereira et al. 2024). Thus, evaluating recognition memory has been proposed as an early predictor of memory-related pathologies. Since many memory disorders begin with recognition deficits, understanding its underlying mechanisms is critical for elucidating and preventing cognitive decline.

A widely used paradigm for investigating recognition memory in rodents is the Novel Object Recognition (NOR) task, based on the innate preference to explore novel stimuli. It consists of two phases: a training phase, during which animals explore two identical unfamiliar objects, and a test phase, in which one object is replaced with a novel one. Rodents display object recognition memory (ORM; the cognitive process) when they spend more time exploring the novel object during the test (Antunes & Biala 2012). Recent studies show that the noradrenergic system (noradrenaline; NA) responds not only to emotionally arousing stimuli (Sara 2009), but also to novel, non-emotional cues (Breton-Provencher et al. 2021; Song et al. 2021; Vankov et al. 1995). Accordingly, multiple lines of evidence support the involvement of NA, and specifically β-adrenergic receptors (β-AR), in recognition memory formation (Balbinot & Haubrich 2023; Barsegyan et al. 2014; Dornelles et al. 2007; Jurado-Berbel et al. 2010; Mello-Carpes et al. 2016; Mello-Carpes & Izquierdo 2013; Moreno-Castilla et al. 2018; Roozendaal et al. 2008; Song et al. 2021). In male rats, systemic or intra-hippocampal post-training

NA administration facilitates ORM when tested 24 hours, 96 hours, or up to 21 days later, indicating a key role in the ORM as assessed in the NOR task (Dornelles et al. 2007; Mello-Carpes et al. 2016; Mello-Carpes & Izquierdo 2013). This effect is blocked by pre-training (Dornelles et al. 2007) or post-training (Mello-Carpes et al. 2016) infusions of $\beta_{1/2}$-AR antagonists propranolol or timolol into the dorsal HIP. Consistent with this, clenbuterol, a β_2-AR agonist, infused into the basolateral amygdala immediately after training, enhances ORM in male rats (McReynolds et al. 2014), further supporting β-AR involvement in consolidation. These findings suggest that NA facilitates ORM via β-AR in male rats. However, the specific role of NA during acquisition in female and male rodents remains unclear, particularly whether NA stimulation at learning is required to initiate long-lasting memory formation. Notably, ORM research has primarily focused on males, with females often excluded or hormonally manipulated to control estrous cycle variability (Becegato and Silva 2022; Nunamaker and Turner 2023). This bias leaves a significant gap in understanding how memory processes, including NA-mediated mechanisms, differ between sexes. Consequently, sex-specific strategies for encoding and processing environmental information remain largely unexplored.

Based on the above, the aim of this investigation was twofold: (1) to assess NA and β_2-AR involvement in recognition memory formation in male and female mice, and (2) to determine whether NA modulation influences neuronal activity in brain regions underlying ORM, namely the HIP and PRH. Although both regions are established substrates of recognition memory, the extent to which NA signaling during acquisition modulates their engagement during retrieval remains unclear. To address this, we quantified c-Fos expression, a marker of neuronal activation, to assess how NA shapes the neural circuits recruited during memory retrieval. We hypothesized that administration of atomoxetine (ATO), a NA reuptake inhibitor, prior to NOR training would facilitate ORM formation in both sexes, whereas blockade of β_2-AR with ICI-118,551 (ICI) would impair it. Furthermore, we predicted that successful ORM would be associated with increased c-Fos expression in the PRH following weak encoding, whereas strong encoding would be associated with increased activation in the CA1 region of the HIP.

METHODOLOGY

Subjects

A total of 93 male and 64 female Swiss mice (Janvier-Labs, St Berthevin Cedex, France) were included in the experiments. Mice were purchased at 4 weeks of age and housed in the animal facilities (Universitat Jaume I) under standard environmental conditions (12-h light cycle from 8:00 am to 8:00 pm or from 1:00 pm to 1:00 am) with access to food and water *ad libitum*. Behavioral procedures were carried out when the mice were between 13 and 16 weeks old. Experiments began two hours after the start of the light cycle. All experiments were conducted in accordance with the guidelines provided by the European Community Council Directive (2010/63/EU), local Animal Welfare Ethics Committee and Empowered Body (CEEA) protocol approval (2023-VSC-PEA-0224), Spanish directive BOE 34/11370/2013, and local directive DOGV 26/2010.

Pharmacological agents

ATO (CAT#Y0001586; Sigma-Aldrich Química, S.L., Madrid, Spain) and ICI (CAT#I127-25MG; Sigma-Aldrich Química, S.L., Madrid, Spain) were dissolved in 0.9% saline (SAL) and administered via intraperitoneal (IP) injection 30 minutes prior to NOR training. The doses used were 3 mg/kg for ATO, 5 mg/kg for ICI, and 0.9% saline as a control vehicle.

NOR task

Objects. Three pairs of identical objects (A, B, or C) were used (Fig. 1a) Two pairs (A and B) were used in Experiments 1, 2, and 3. Objects A and B were similar in size and color (both pink; A: 13 × 5 × 5 cm; B: 13.5 × 5 × 5 cm) but differed in texture. Object A had a smooth surface, whereas object B featured a rough texture with several bumps. For Experiments 3, 4, and 5, objects A and C were used. Object C differed markedly from both A and B in several characteristics, including size (C: 15 × 5 × 1.5 cm), color (red instead of pink), shape, and texture (Fig. 1a). To ensure that animals did not show an innate preference for any object, a preference test was performed in naïve male (n = 4) and female mice (n = 4). Mice were presented with two objects during a 5-min trial, and exploration time was recorded. Discrimination scores were close to zero for both sexes, indicating no inherent preference. The familiar object (A, B, or C), as well as its position during testing (left or right relative to the center of the chamber), was counterbalanced across animals.

Protocols. Behavioral experiments were conducted in a black opaque enclosure (30 × 30 × 30 cm). Before starting the experimental procedures, animals were habituated to the experimental room for 30 minutes to reduce stress. Each experiment consisted of three phases: habituation, training, and test. During habituation, animals were weighed and exposed to the enclosure for 5 minutes, allowing them to explore the arena without any objects present. Twenty-four hours later, the training phase took place, during which animals were free to explore two identical objects (AA, BB, or CC). All pharmacological agents were administered 30 minutes prior to training. This timing was based on previous literature demonstrating that administration of ATO or ICI at this time point influences memory formation (Fitzgerald et al. 2016; Montezinho et al. 2010; Stapley et al. 2013). To assess ORM, animals were presented with two objects - a familiar and a novel one (AB, BA, AC, CA) - and allowed to explore them for 5 minutes. Object configurations were counterbalanced across groups as follows: in Experiment 1, SAL (n = 12 AB, n = 11 BA) and ATO (n = 11 AB, n = 10 BA); in Experiment 2, SAL (n = 3 AB, n = 7 BA) and ICI (n = 5 AB, n = 5 BA); in Experiment 3, A vs B (n = 8 AB, n=8 BA) and A vs C (n = 5 AC, n = 5 CA); in Experiment 4, SAL (n = 5 AC, n = 4 CA) and ATO (n = 5 AC, n = 5 CA); and in Experiment 5, SAL (n = 6 AC, n = 4 CA) and ICI (n = 6 AC, n = 4 CA). Training and test sessions were videorecorded to analyze exploration time for both objects as a measure of ORM. All NOR procedures were adapted from Cruz-Sanchez et al. (2021), de Bruin & Pouzet (2006), Leger et al. (2013), and Moura et al. (2006). To assess the effects of NA manipulations on NOR in both sexes, five separate experiments with varying timing protocols were conducted. The duration of the training phase and the interval between training and testing were adjusted across experiments (see timeline for each experiment) (Fig. 2, 5-7).

Experiment 1. This experiment investigated whether treatment with the NA reuptake inhibitor ATO enables memory acquisition under conditions designed to produce subthreshold memory traces. A protocol was designed in which the control group (males and females) was expected to show a discrimination index (DI) close to zero, indicating little or no memory of the familiar object. On the training day, 30 male and 43 female mice were administered SAL (n = 15 males, n = 23 females) or ATO (3 mg/kg; n = 15 males, n = 21 females) and allowed to explore two identical objects for 1 minute. A 5-minute test was conducted 48 hours later, and brains were collected 60 minutes after the test.

Experiment 2. Building on Experiment 1, which revealed a lack of ORM acquisition in males, this experiment focused on female mice to examine the role of β_2-AR. The aim was to determine whether β_2-AR blockade with ICI interferes with ORM acquisition in female mice (n = 20). A NOR protocol in which control animals typically achieve a high DI was used. Female mice were trained for 10 minutes, 30 minutes after administration of saline or ICI (5 mg/kg). A 5-minute test was conducted 24 hours later, and brains were collected 60 minutes after the test for c-Fos analyses.

Experiment 3. Male mice have difficulty distinguishing highly similar objects, which may explain the lack of discrimination observed in Experiment 1. This experiment tested whether the absence of ATO's effect in males was due to reduced object discrimination ability. Twenty-six male mice were trained in a drug-free state for 5 minutes and tested 1 hour later using either objects similar in length and color but differing in shape and texture (AB; n = 16), or objects differing in multiple features, including size, color, shape, and texture (AC; n = 10). Successful short-term ORM would indicate the animals could discriminate between the objects. Previous studies show that female mice can discriminate highly similar objects, whereas males often fail (Bertaina-Anglade et al. 2006; Bettis & Jacobs 2012, 2013;Mariscal et al. 2023; Moura et al. 2006).

Experiment 4. Using a set of sufficiently dissimilar objects that male mice could reliably discriminate under short-term memory conditions, we tested whether ATO could promote long-term ORM in males (n = 19). The procedure matched Experiment 1 in females: a 1-minute training session followed by a 48-hour interval before testing, using the newly selected, more dissimilar objects. This design assessed whether noradrenergic modulation via ATO enables long-term memory acquisition in male mice under conditions that normally do not support ORM.

Experiment 5. This experiment examined whether β_2-AR blockade with ICI interferes with ORM acquisition in male mice (n = 18). A NOR protocol in which control animals typically achieve a high DI was used. Male mice were trained for 10 minutes, 30 minutes after administration of saline or ICI (5 mg/kg). A 5-minute test was conducted 24 hours later.

Video analyses. All videos were analyzed by a blind observer. To be considered exploratory behavior, animals were required to keep their nose within 1 cm of the object, regardless of whether they were positioned above it. Behaviors such as biting the object or sitting on top of it while scanning the environment were not classified as exploration.

Brain sampling. Animals from Experiments 1 and 2 were transcardially perfused 60 minutes after the NOR test using a heparin solution (0.006%; Heparin sodium salt from porcine intestinal mucosa, CAT#H3393; Sigma-Aldrich, Madrid, Spain) dissolved in 0.9% saline, followed by 4% paraformaldehyde (Paraformaldehyde powder, 95%, CAT#158127; Sigma-Aldrich, Madrid, Spain) after anesthesia with sodium pentobarbital (20 mg/kg; Dolethal 100 mL, Vetoquinol E.V.S.A., Madrid, Spain). Samples were fixed in 4% paraformaldehyde at room temperature for 24 hours and then stored in 30% sucrose with 2% sodium azide (Sodium azide, CAT#1.06688.0100; Merck, Darmstadt, Germany) at 4°C. Coronal brain slices (40 μm) were obtained using a cryostat microtome (Microm HM560, Thermo Fisher Scientific, Barcelona, Spain) and stored at −20°C in cryoprotectant solution.

Immunofluorescence. c-Fos immunofluorescent staining was used to evaluate neural activity. Free-floating slices were incubated for 24 hours at 4°C with rabbit anti-c-Fos (1:2000, CAT#226308, Synaptic Systems, Göttingen, Germany) in PBST. Subsequently, tissue was incubated for 2 hours with donkey anti-rabbit Alexa Fluor 555 (1:250, CAT#A-31572, Thermo Fisher Scientific, Barcelona, Spain) in PBST.

Image acquisition and analysis. Z-stack images of the CA1, CA3, DG, and PRH (Fig. 1b) were acquired using a confocal microscope at the Scientific Instrumentation Service (SCIC) of the Universitat Jaume I (Leica DMi8, Leica Microsystems CMS GmbH, Wetzlar, Germany). Maximal projections of Z-stacks were generated using Leica Application Suite X (LAS X, Leica Microsystems CMS GmbH, Wetzlar, Germany). c-Fos$^+$ cells were analyzed using Fiji software (Schindelin et al., 2012). To ensure reproducibility, two brain sections per hemisphere were selected for analysis, resulting in a total of four images per animal. In a few cases, only three images were available due to tissue damage. Sections were collected at bregma levels ranging from −1.58 to −2.06 mm. Region-of-interest (ROI) dimensions varied by region: CA1 (563 × 242 μm), CA3 (517 × 521 μm), DG (580 × 354 μm), and PRH (517 × 521 μm).

Experimental design and statistical analysis. R statistical language (version 4.2.2; R Core Team, 2022) was used for most analyses, and GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA, USA) was used for Chi-squared tests (χ^2) and figure preparation. Normality of the data was assessed using the Shapiro-Wilk test, which indicated non-normal distributions in some groups. Therefore, non-parametric statistics were used for all analyses. These methods require fewer assumptions about data distribution and rely on robust measures of central tendency, such as the median, making them suitable for small sample sizes and less sensitive to outliers.
Object recognition memory (ORM) performance was quantified using the discrimination index (DI), which ranges from −100 to +100, with zero indicating chance-level performance. Positive DI values reflect greater exploration of the novel object, whereas negative values indicate a preference for the familiar object. DI was calculated as follows:

$$DI = \frac{Time\ exploring\ NEW - Time\ exploring\ FAMILIAR}{Total\ time}\ x\ 100$$

To compare DI between independent groups we used the shifthd_pbci (bootstrap, 5000 reshuffles) function from the *rogme* package (Rousselet et al. 2017). P-values were

reported, and when $p<0.05$, we also provided the difference between medians (Δ_{Median} = Median 1 – Median 2) as a measure of effect size, along with 95% confidence intervals (CI) for that difference. To explore correlations between DI and c-Fos+ cell counts, Kendall's tau correlation coefficient (tau) was calculated (Kendall 1938). Tau values range from -1 to 1, where values close to 1 indicate a strong positive correlation, and values close to -1 indicate a strong negative correlation. The magnitude of the correlation increases as tau moves further from 0. P-values and 95% CI of tau coefficient were also reported. χ^2 test was analyzed to explore frequency distribution of positive and negative DI among groups.

RESULTS

Experiment 1. Noradrenergic stimulation of ORM in male and female mice: Behavioral and c-Fos studies

During training, females spent less time exploring objects when compared to males. Since animals were trained under the influence of ATO, we aimed to determine whether this drug affected the time they spent exploring during training. No significant differences were observed between treatment groups (SAL vs. ATO) in either males ($p=0.51$) or females ($p=0.55$). Given the absence of treatment effects, and in order to investigate potential sex differences, data from SAL and ATO groups were combined, and males were compared to females irrespective of treatment condition. Our data analyses showed that females spent less time exploring objects than males during the 1-minute training session [$p<0.0001$; $\Delta_{MALES-FEMALES} = 564.54$; 95% CI = (287.63, 917.41)]. During the 5-minute test, no significant effect of treatment was observed on the time spent exploring objects in either males ($p=0.23$) or females ($p=0.82$). Additionally, no significant sex differences were detected ($p=0.097$) (Fig. 2b, c). These findings suggest that, regardless of treatment, there may be inherent sex-related tendencies in the exploration of environmental stimuli over short durations. Note that for both; training and testing, all four groups were displayed separately (SAL males, ATO males, SAL females, and ATO females).

On the test day, ATO facilitates ORM in female but not in male mice. To explore noradrenergic modulation of ORM via NA reuptake inhibitor, separate analyses were performed for males and females. Regarding males, no significant differences in the DI were found between the SAL and ATO groups ($p=0.41$). Similarly, the frequency distribution of positive and negative DI values did not differ between groups ($\chi^2=0.536$, $p=0.46$). In contrast to males, analyses in females revealed a significant effect of the DI [$p<0.0001$; $\Delta_{SAL-ATO} = -27.53$; 95% CI = (-38.41, -14.07)]. Follow-up analysis of data distribution revealed that ATO significantly increased the proportion of females exhibiting positive DI values compared to SAL ($\chi^2 = 5.50$, $p = 0.019$), an effect not observed in males (Fig. 2e, f). These results suggest that noradrenergic stimulation facilitates ORM in females, but not in males following a procedure involving 1-min exploration training.

Females that received ATO during training showed increased c-Fos activity in the PRH during the test compared to control animals. Hippocampal and PRH involvement during the NOR test was assessed using c-Fos as a proxy to neural activity. We explored hippocampal areas (CA1, CA3 and DG) and PRH in both males and females. Independent comparisons showed no differences in any area in males (CA1, $p=0.36$;

CA3, p=0.51; DG, p=0.23; PRH, p=0.55) (Fig. 3). However, females receiving ATO before training showed higher c-Fos activity in PRH during test relative to the SAL group [p=0.046; $\Delta_{SAL-ATO}$ = -52.08; 95% CI = (-84.65, -2.26)]. On the contrary, no differences were observed in any of the hippocampal regions evaluated (CA1, p=0.99; CA3=, p=0.33; DG, p=0.44) (Fig. 4), further suggesting that noradrenergic modulation may influence the recruitment of the PRH. The Kendall rank correlation coefficient (*tau*) was calculated to assess the relationship between DI and c-Fos activity; however, no significant correlation was found (Table 1).

Experiment 2. β_2-AR blockade during ORM in female mice: Behavioral and c-Fos studies

ICI prevents ORM acquisition in female mice. As in previous studies, we first evaluated the effects of the β_2-AR antagonist ICI during training. A comparison for independent groups yielded no differences in time exploring objects between SAL and ICI groups during either training (p=0.74) or test sessions (p=0.59) (Fig. 5b).
To evaluate the effect of β_2-AR blockade on ORM, a comparison of independent groups revealed a significant difference in the DI on the test day between females receiving SAL or ICI 30-min prior to training [p=0.033; $\Delta_{SAL-ICI}$=18.90;95% CI = (0.96,33.22)] (Fig. 5c). While the $\chi 2$ test did not indicate a significant difference in the frequency distribution ($\chi 2$=2.4, p=0.121), descriptive analysis showed that only 60% of the animals in the ICI group displayed a positive DI, compared to 90% of the control group (Fig. 5d). Collectively, these data suggest that β_2-ARs are necessary for the formation of ORM in females.

c-Fos activity in the HIP and PRH was not affected by ICI administration. No differences in c-Fos expression were observed between SAL and ICI groups in hippocampal regions (CA1, p=0.62; CA3, p=0.48; DG, p=0.66) and the PRH (p=0.65) (Fig. 5e-h). Furthermore, no correlations were found between DI and c-fos activity in any region, as assessed using Kendall's rank correlation coefficient (*tau*) (Table 2).

Experiment 3. Assessment of short-term ORM in drug-free male mice: Comparing similar and dissimilar objects

Male mice exhibit short-term ORM for dissimilar, but not similar, objects. Male mice displayed a significantly higher DI when presented with dissimilar objects (AC) compared to similar objects (AB) [p<0.0001; Δ_{AB-AC}=-35.06; 95% CI = (-53.44, -12.07)] (Fig. 6b). A $\chi 2$ analysis further confirmed a significant difference in the frequency distribution of DI scores between the two conditions (χ^2=0.35, p=0.037) (Fig. 6c). These data indicate that while male mice successfully exhibit ORM when distinct stimuli are used, they struggle to discriminate between objects with high structural similarity. This suggests a higher threshold for stimulus differentiation in males, as they require more salient physical differences to guide recognition memory. Consequently, the AC object pair was selected for all subsequent NOR experiments involving male cohorts.

Experiment 4. Noradrenergic stimulation of ORM in male mice using dissimilar objects

ATO administration fails to facilitate ORM in male mice. Systemic administration of

ATO prior to ORM training did not significantly facilitate memory performance in male mice ($p=0.25$). Consistent with these results, a $\chi 2$ analysis revealed no significant differences in the frequency distribution of DI scores between groups ($\chi 2=1.35$, $p=0.24$). These findings indicate that augmenting endogenous NA levels via reuptake inhibition does not further enhance long-term ORM in males when using highly discriminable stimuli (Fig. 7b, c).

Experiment 5. β_2-AR antagonism of ORM in male mice using dissimilar objects

β_2-AR blockade does not impair ORM in male mice. ORM was not affected by blocking β_2-AR prior to NOR training in male mice ($p=0.08$). Consistent with this, a $\chi 2$ test revealed no differences in the frequency distribution between the control and treatment groups ($\chi 2=0$, $p>0.99$). These results indicate that β_2-AR antagonism fails to impair long-term ORM in males, suggesting that noradrenergic signaling via these receptors is not a requirement for object recognition memory in this sex (Fig. 7e, f).

DISCUSSION

In this investigation, we aimed to characterize: (1) exploratory behavior patterns in male and female mice during the training and testing phases of the NOR task; (2) the contribution of NA and β_2-AR to ORM; and (3) the neural correlates underlying ORM recall. Regarding the first objective, two primary findings emerged: during a 1-minute training session, females explored the identical object pair less than males. However, this increased exploration in males did not translate into more accurate memory encoding; notably, males required more dissimilar objects to achieve significant discrimination in both short- and long-term memory tasks. Furthermore, following a 1-minute training session and a 48-hour delay, administration of ATO 30 minutes prior to training enabled ORM formation in females but not in males, even when more dissimilar objects were used. This effect was accompanied by increased c-Fos activity in the PRH during the NOR test, observed exclusively in ATO-treated females. Conversely, ORM formation was abolished in females when the β_2-AR antagonist ICI was administered prior to training, whereas no such effect was observed in males. Additionally, no significant differences in c-Fos were detected across the examined brain regions in either sex. These results highlight: (1) sex-specific patterns in how mice interact with and learn about their environment; (2) that NA acting via β_2-AR is essential for ORM consolidation in females; and (3) that PRH activity is selectively recruited during ORM expression in females following NA-dependent training. In contrast, the HIP does not appear critical for memory retrieval under our experimental conditions.

Sex-specific patterns of exploration and stimulus processing in Swiss mice during a NOR task

Evidence on sex differences in exploratory behavior and object recognition in the NOR task is mixed. Some studies report no differences in mice (Benice et al. 2006; Podhorna & Brown 2002) or rats (Becegato & Silva 2022; Chuhan & Taukulis 2006; Saucier et al. 2007a), whereas others describe sex-dependent effects, with females showing reduced object exploration (Mariscal et al. 2023; Melgar-Locatelli et al. 2024). Consistent with the latter, our findings reveal two distinct profiles: during a 1-minute training session, females explored identical objects less than males yet performed better in the NOR test.

Increased exploration in males did not enhance discrimination, and short-term memory data show that males require greater object dissimilarity to detect novelty, whereas females can discriminate highly similar objects. This may explain why sex differences in total exploration are not always evident during testing. These results align with prior reports showing that females outperform males in discriminating subtly different objects in mice (Bettis & Jacobs 2012, 2013; Mariscal et al. 2023), rats (Saucier et al. 2007a), and humans (Saucier et al. 2007b; Voyer et al. 2007). One explanation is that males and females adopt different information-sampling strategies: males explore both spatial and visual features and shift behavior frequently, whereas females rely on a stable, location-focused strategy that promotes efficient encoding despite less overall interaction (Chen et al. 2021). Females may focus on fine-grained object features, while males depend more on spatial cues (Saucier et al. 2007a, b). Detecting novelty engages attentional and arousal systems, suggesting these behavioral differences reflect underlying neurobiological dimorphism in the locus coeruleus (LC) (Sara 2009). Although LC structure and neuron counts vary across species and strains (Bangasser et al. 2011; Garcia-Falgueras et al. 2005; Mariscal et al. 2023; Pinos et al. 2001; Sun et al. 2020), females consistently show greater dendritic complexity and excitability in LC NA neurons (Mariscal et al. 2023), supporting more efficient processing of fine sensory details. Variations in locomotor activity may also contribute to sex differences in memory recognition, but evidence is inconsistent. Some studies report higher activity in females (Borbélyová et al. 2019; McElroy & Howland 2025), whereas others find no sex differences during NOR (Melgar-Locatelli et al. 2024). Sex differences in object exploration persist even in longer sessions (Mariscal et al. 2023; Melgar-Locatelli et al. 2024), suggesting that reduced exploration in females reflects strategy rather than locomotion. Overall, these findings highlight that memory formation is shaped by sex-specific exploratory and encoding strategies, underscoring the importance of including females in memory research.

The role of NA and β_2-AR in ORM formation in females and males

Our findings show that enhancing NA signaling via ATO facilitates ORM formation in female mice, consistent with prior work showing that ATO administration before training improves ORM in rats (Tzavara et al. 2006). Importantly, whereas previous studies described ATO effects as modulatory, our data indicate that NA can be necessary for memory formation under conditions in which animals would otherwise fail to exhibit ORM. Complementary evidence supports a role for NA in long-term memory: post-training systemic or intra-basolateral amygdala infusions of NA or the β_2-AR agonist clenbuterol enhance ORM for days to weeks (Barsegyan et al. 2014; Dornelles et al. 2007; McReynolds et al. 2014; Mello-Carpes et al. 2016). At the neurobiological level, ATO (3 mg/kg) induces a gradual, sustained increase in NA lasting up to 180 minutes (Ago et al. 2014; Koda et al. 2010), likely facilitating synaptic plasticity during NOR training. NA modulates long term potentiation via β-AR (Carey & Regehr 2009; Sara 2009), suggesting that ATO enhances ORM primarily through β_2-AR-dependent facilitation of plasticity mechanisms. Other strategies that elevate NA, such as α_2-AR antagonism, also enhance ORM (Song et al. 2021). Blocking β_2-AR with ICI prevented ORM formation in females, highlighting a specific role for β_2-AR signaling in memory encoding. These results complement prior findings showing that non-selective $\beta_{1/2}$-AR antagonism disrupts consolidation and reconsolidation of ORM (Balbinot & Haubrich 2023; Dornelles et al. 2007; Liu et al. 2015; Mello-Carpes et al. 2016; Song et al. 2021. In the HIP, β-AR signaling supports object-spatial associations

and novelty-induced neuronal activity (Balbinot & Haubrich 2023; Kitchigina et al. 1997), reinforcing NA's central role across memory phases.

Behavioral effects observed in females were absent in males under identical conditions. The ATO dose used in our study (3 mg/kg) falls within ranges previously reported to enhance ORM, spatial memory, and extinction of cocaine self-administration in males (Callahan et al. 2019; Janak et al. 2012; Tzavara et al. 2006), and ICI doses (1-8 mg/kg) impair reconsolidation of male CPP (Bernardi et al. 2009; Fitzgerald et al. 2016). The null effect in males may reflect dose-dependent variability, consistent with the inverted U-shaped relationship between NA and cognition, where both insufficient and excessive activity can impair function (Cools & Robbins 2004; Holland et al. 2021). Baseline differences, with females outperforming males, may contribute but do not fully explain why ATO improved females while males showed inconsistent responses. These sex-specific effects align with evidence that males and females rely on distinct β-AR subtypes and neural circuits across memory stages. In CPP paradigms, males show broad β_1- and β_2-AR involvement across learning, retrieval, and retention, whereas females display subtype-specific roles, with β_2-AR supporting acquisition and consolidation and β_1-AR mediating retrieval. The lack of effect in males after selective β_2-AR blockade may be due to compensatory activity of other adrenergic receptors, whereas females rely more directly on β_2-AR, making their performance more sensitive to its modulation (Berry et al. 2024). Taken together, these findings support fundamental sex differences in LC-NA system sensitivity and regulation (Mariscal et al. 2023; Sun et al. 2020). Females' greater responsiveness to both ATO facilitation and ICI disruption may also reflect higher dendritic complexity, density, and excitability of LC neurons (Mariscal et al. 2023), supporting efficient processing of fine sensory details. Behavioral differences may additionally reflect distinct exploratory strategies: females adopt a stable, location-focused approach prioritizing fine-grained object features, whereas males rely more on spatial cues and frequently shift attention (Chen et al. 2021; Saucier et al. 2007a, b).
Overall, our results highlight that NA, acting via β_2-AR, is critical for ORM formation in females, whereas males may require different receptor engagement or higher NA levels to achieve comparable effects. These findings reinforce the importance of including both sexes in cognitive research, as sex-specific differences in NA modulation and exploratory behavior substantially influence ORM. Future studies should investigate receptor expression, signaling dynamics, and NA turnover as mechanisms underlying these sex divergences.

The involvement of PRH in ORM

Our findings showed increased c-Fos expression in the PRH of ATO-treated females during the NOR test, with no changes in the HIP. This aligns with the functional specialization of these regions: PRH primarily encodes object identity and novelty, whereas HIP integrates spatial and contextual information (Arias et al. 2015; Chao et al. 2022; Méndez et al. 2015; Ward 2019). HIP engagement in NOR is usually observed when object exploration involves spatial rearrangements (Albasser et al. 2010; Piterkin et al. 2008; Yi et al. 2016). Thus, the lack of HIP activation likely reflects minimal spatial demands, whereas PRH activity supports object processing. Notably, males showed no PRH activation, paralleling the absence of behavioral effects.
Despite elevated PRH c-Fos expression in ATO-treated females, no correlation with DI was observed. Although the discrimination index is a behavioral measure of memory

performance, it reflects the integration of multiple processes, including exploration, motivation, and decision-making, which may not map linearly onto regional neuronal activity. In contrast, c-Fos provides a coarse measure of population-level activation and may not capture the specific neuronal ensembles that directly support successful discrimination. Notably, previous studies have shown that PRH activity correlates with short-term NOR performance but not long-term recognition (Albasser et al. 2010; Méndez et al. 2015), consistent with the 48-hour delay used here. During training, PRH encodes sensory and associative object features, whereas testing recruits retrieval, novelty detection, and concurrent encoding of new information, potentially involving partially distinct neuronal populations (von Linstow Roloff et al. 2016; Winters et al. 2008; Winters & Bussey 2005; Zhu & Brown 1995). Thus, bulk c-Fos measured at test may primarily reflect retrieval- and novelty-related processes rather than discrimination performance per se. Importantly, the absence of correlation does not preclude a functional role of the PRH but instead suggests that its contribution to ORM is necessary yet not solely predictive of behavioral output at the individual level. This interpretation is consistent with a distributed network model in which PRH supports object representations, while successful performance emerges from interactions across multiple regions.

In the ICI experiment, SAL and ICI groups exhibited similar PRH c-Fos expression, with no correlation to DI, consistent with differences in memory strength rather than task failure. Recognition memory is increasingly viewed as a continuum from weaker familiarity to stronger recollection (Bastin et al. 2019; Eichenbaum & Lipton 2008; Squire et al. 2007; Wiesmann 2008). PRH preferentially supports weaker traces, whereas HIP is recruited for stronger representations (Cinalli et al. 2020; Ward 2019). Supporting this, PRH engagement was more evident under limited exploration (Exp. 1) and diminished with extended exploration (Exp. 2), consistent with c-Fos reflecting retrieval rather than discrimination. The lack of HIP activation contrasts with reports of CA1 c-Fos increases following novel object exposure (Cinalli et al. 2020), suggesting hippocampal involvement depends on contextual or spatial demands
Overall, these results indicate that ORM relies on NA signaling via β_2-AR and shows clear sex-specific effects. ATO facilitated ORM and enhanced PRH activity in females, whereas β_2-AR blockade impaired memory formation. PRH activation appears to reflect retrieval of weaker ORM rather than discrimination per se, while hippocampal engagement may depend on spatial or contextual demands. Collectively, ORM arises from dynamic interactions between PRH and HIP, modulated by NA tone, with LC-PRH connectivity representing a promising avenue for future research.

Conclusions

In summary, our findings demonstrate that sex-specific exploratory strategies, combined with NA signaling via β_2-AR, critically shape ORM. Females efficiently encode fine-grained object information and benefit from enhanced NA tone, whereas males require greater dissimilarity or possibly distinct receptor engagement to achieve comparable memory performance. PRH activity supports retrieval of weaker ORM, while HIP involvement appears contingent on spatial or contextual demands. These results underscore the importance of including both sexes in memory research and highlight the LC-NA-PRH pathway as a key mechanism underlying sex-dependent object recognition memory.

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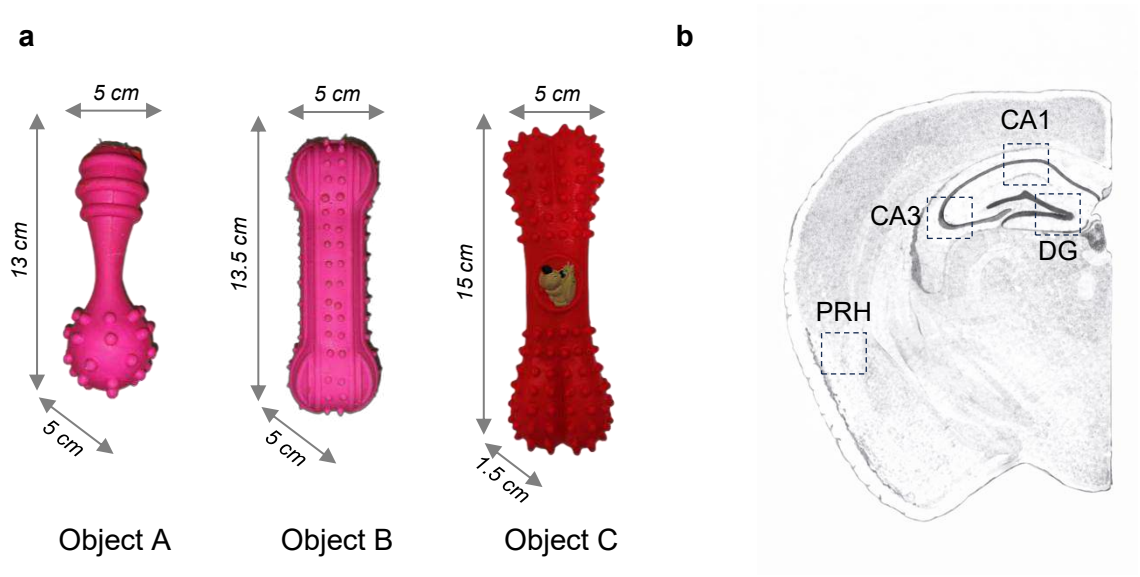


Fig. 1 (a) Objects (A, B, C) used in the Novel Object Recognition task and corresponding schematics with dimensions (length × width × height, cm). **(b)** Representative coronal brain section illustrating regions of interest for c-Fos immunoreactivity assessment: hippocampal subregions CA1 and CA3, dentate gyrus (DG) and perirhinal cortex (PRH)

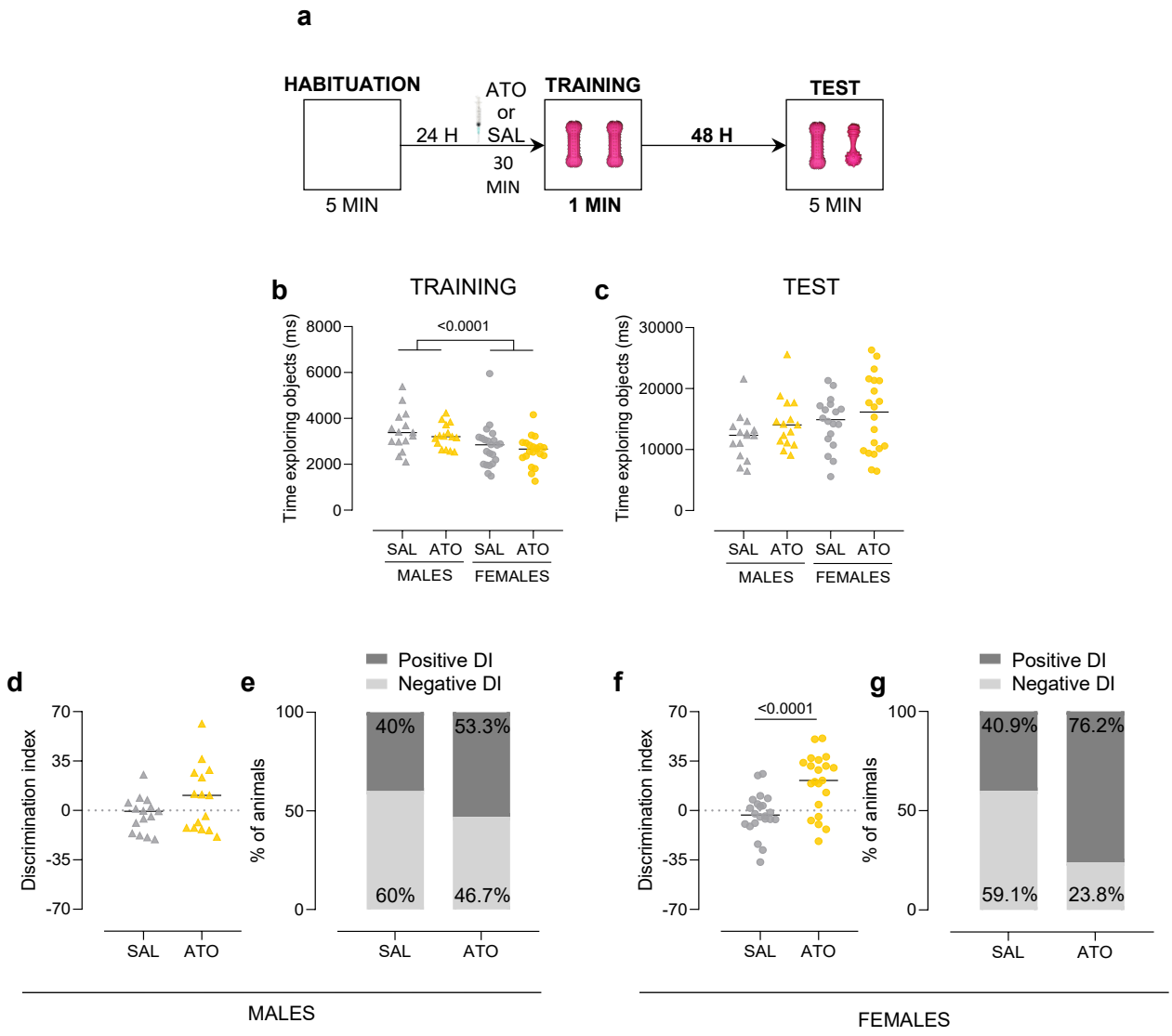


Fig. 2 (a) Novel Object Recognition (NOR) protocol for Experiment 1 (short training, long training-test interval; males and females): timeline showing experimental phases, session durations, and inter-session intervals. **(b, c)** Object exploration time during training and test phases. Total exploration time for both objects is expressed in milliseconds (ms). Data are presented as individual values (scatterplots) with medians indicated. **(d, e)** Discrimination index (DI) and frequency distribution of DI during the NOR test in males and **(f, g)** females. DI is shown as scatterplots of individual values with group medians (ATO, atomoxetine; SAL, saline). Stacked bar charts represent the percentage of animals in positive and negative DI categories. P values are indicated when $p < 0.05$

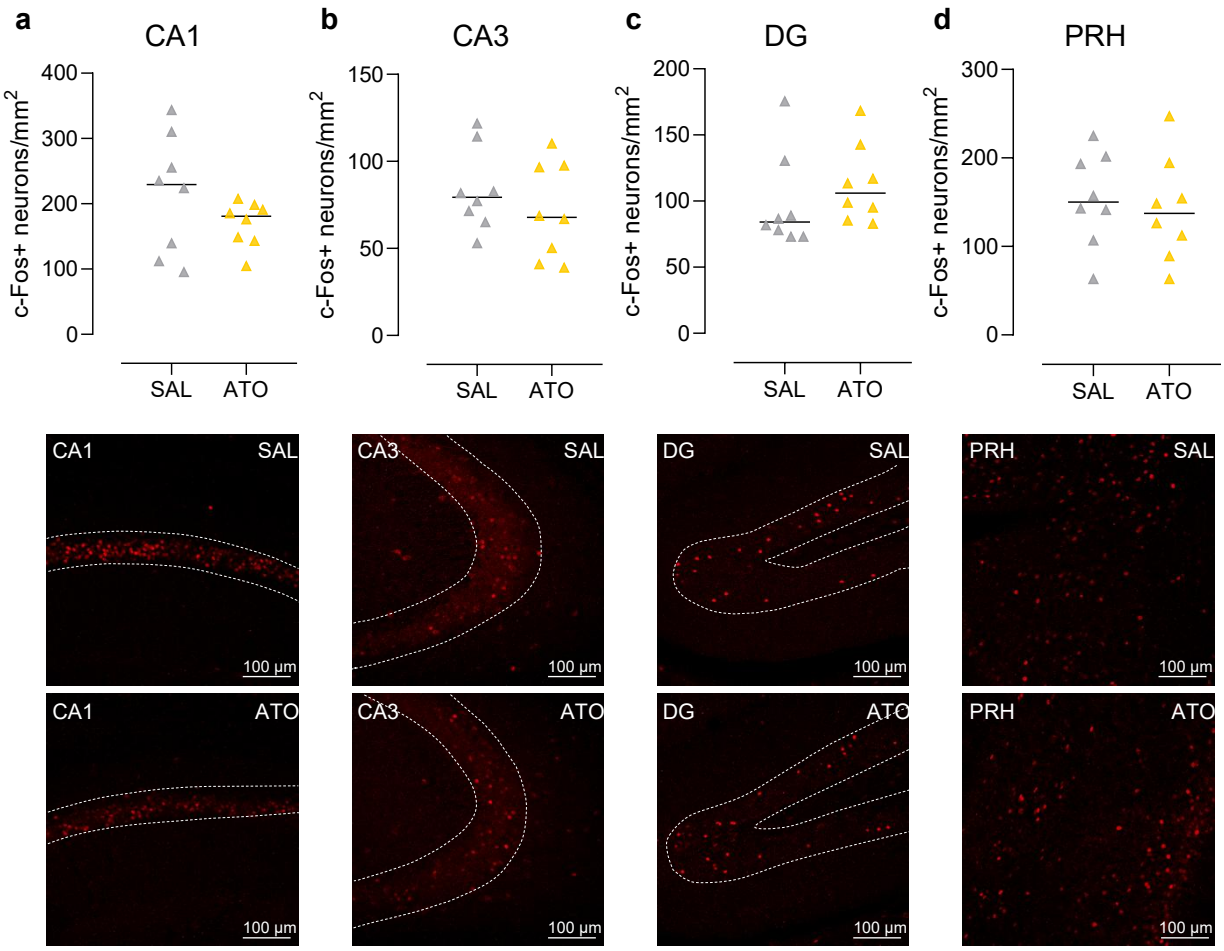


Fig. 3 (a, b, c, d) c-Fos expression in hippocampal subregions CA1 and CA3, dentate gyrus (DG), and perirhinal cortex (PRH) in male subjects. Data are presented as scatterplots showing the number of c-Fos+ neurons/mm² for individual animals, with group medians indicated (ATO, atomoxetine; SAL, saline). P values are shown when $p < 0.05$. Representative images of c-Fos+ cells for each region are shown below (scale bar = 100 µm)

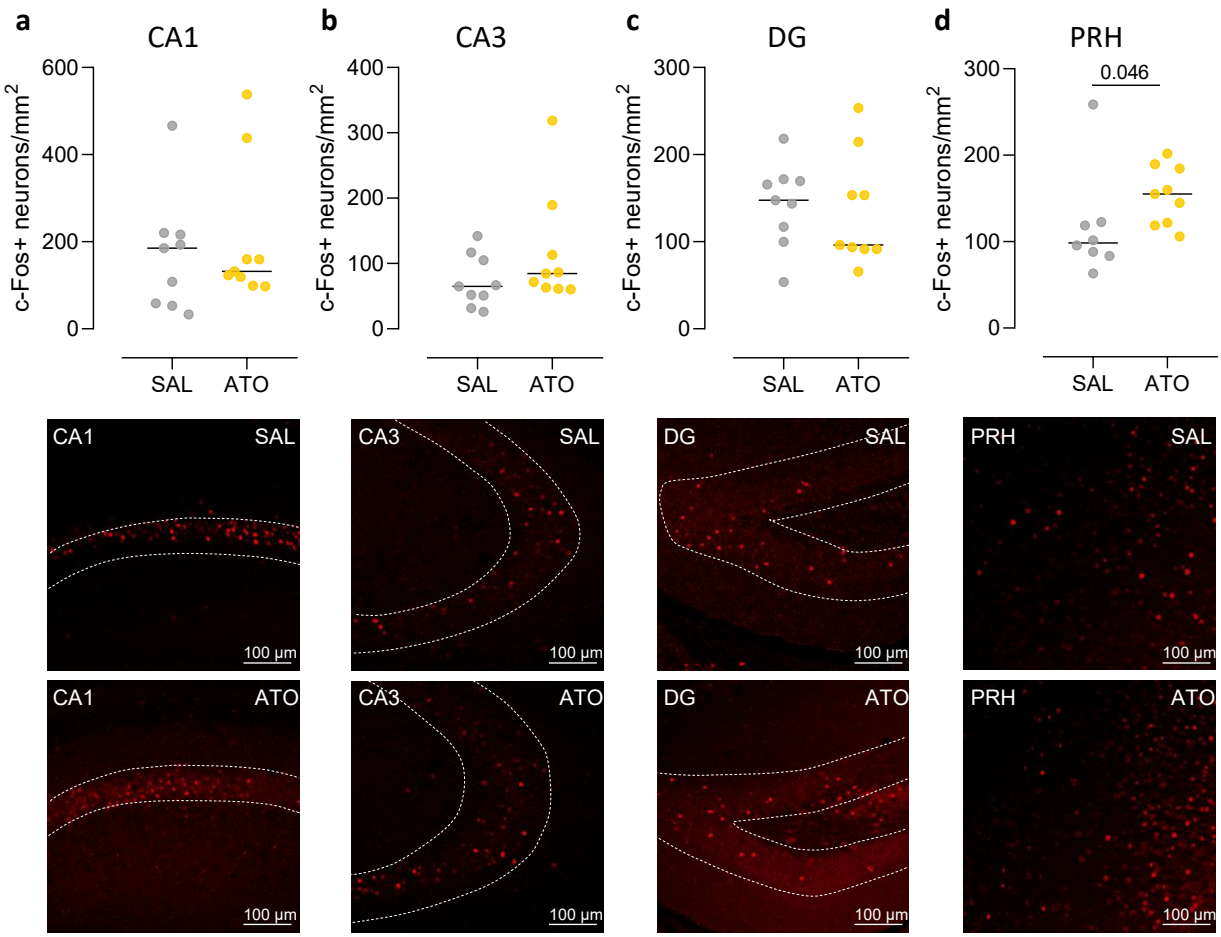


Fig. 4 (a, b, c, d) c-Fos expression in hippocampal subregions CA1 and CA3, dentate gyrus (DG), and perirhinal cortex (PRH) in female subjects. Data are presented as scatterplots showing the number of c-Fos+ neurons/mm² for individual animals, with group medians indicated (ATO, atomoxetine; SAL, saline). P values are shown above graphs when $p < 0.05$. Representative images of c-Fos+ cells for each region are shown below (scale bar = 100 µm)

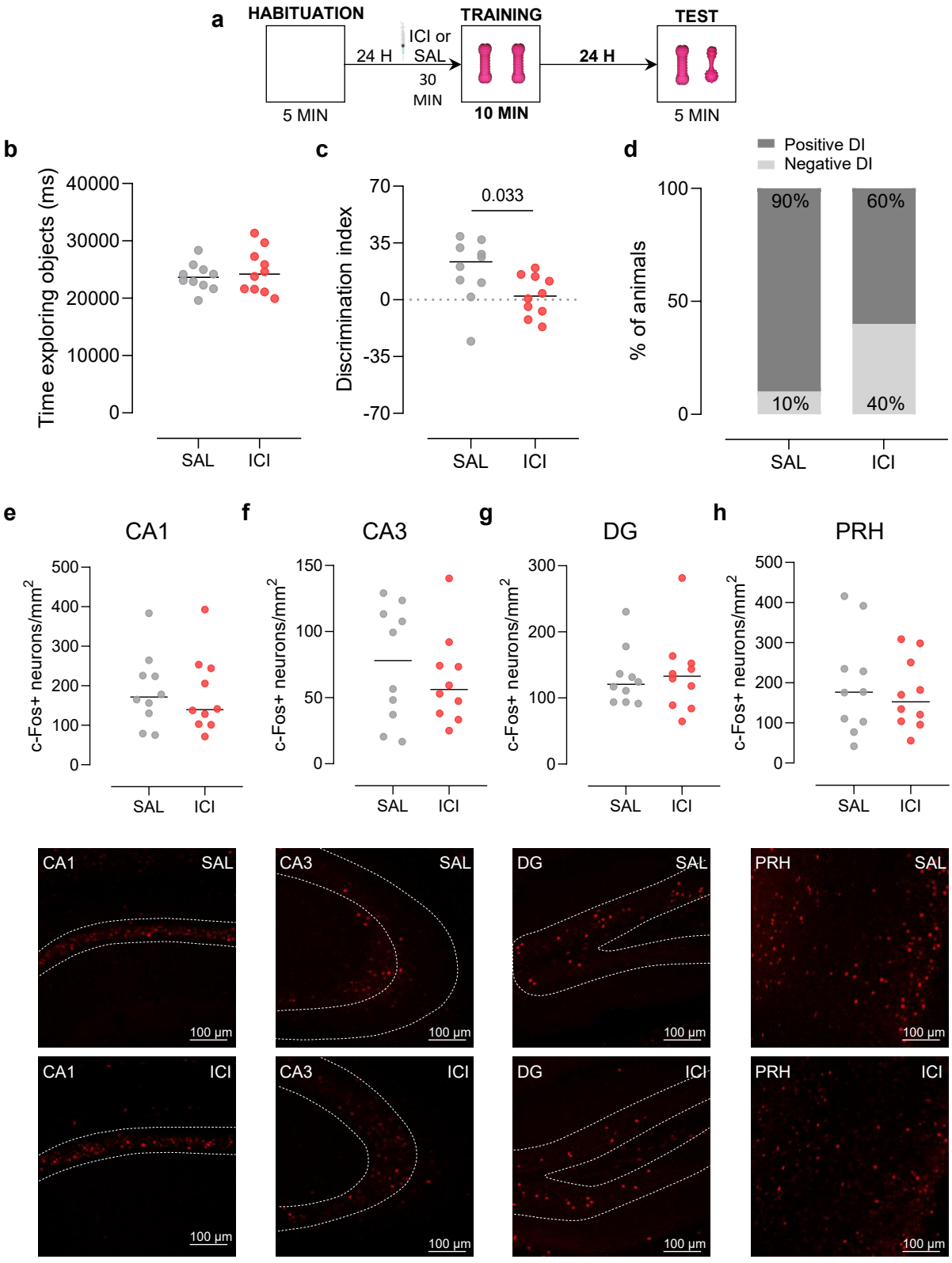

a
HABITUATION
5 MIN
24 H
ICI or SAL
30 MIN
TRAINING
10 MIN
24 H
TEST
5 MIN
b
Time exploring objects (ms)
40000
30000
20000
10000
0
SAL
ICI
c
Discrimination index
70
35
0
-35
-70
0.033
SAL
ICI
d
Positive DI
Negative DI
% of animals
100
50
0
90%
10%
60%
40%
SAL
ICI
e
CA1
c-Fos+ neurons/mm2
500
400
300
200
100
0
SAL
ICI
f
CA3
c-Fos+ neurons/mm2
150
100
50
0
SAL
ICI
g
DG
c-Fos+ neurons/mm2
300
200
100
0
SAL
ICI
h
PRH
c-Fos+ neurons/mm2
500
400
300
200
100
0
SAL
ICI
CA1 SAL
CA3 SAL
DG SAL
PRH SAL
CA1 ICI
CA3 ICI
DG ICI
PRH ICI
100 µm

Fig. 5 (a) Novel Object Recognition (NOR) protocol for Experiment 2 (long training, short training-test interval; females only): timeline showing experimental phases, session durations, and inter-session intervals. **(b)** Total object exploration time during training and test (ms). Data are presented as individual values (scatterplots) with group medians indicated. **(c)** Discrimination index (DI) during the NOR test: data are shown as individual values with group median (SAL; saline or ICI; ICI-118,551). **(d)** Frequency distribution of test DI: stacked bar charts represent the percentage of animals displaying positive or negative DI by treatment group. **(e, f, g, h)** c-Fos expression in hippocampal subregions CA1 and CA3, dentate gyrus (DG), and perirhinal cortex (PRH): data are presented as scatterplots showing the number of c-Fos+ neurons/mm² for individual animals, with group medians indicated. P values are when $p < 0.05$. Representative images of c-Fos+ cells for each region are shown below (scale bar = 100 μm)

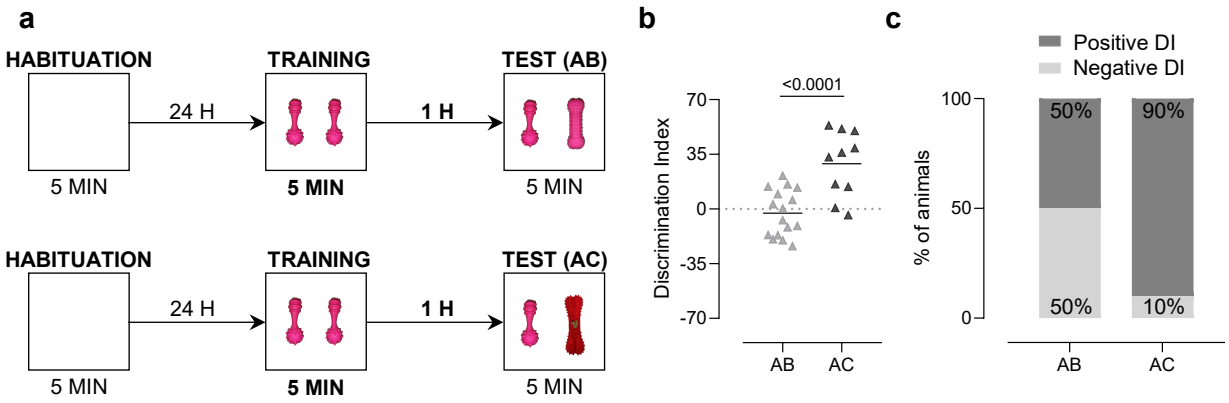


Fig. 6 (a) Novel Object Recognition (NOR) protocol for Experiment 3 (short-term memory protocol; males only), with a timeline showing the objects used, experimental phases, session durations, and inter-session intervals. **(b)** Discrimination index (DI) during the NOR test following a short-term memory protocol. Data are presented as individual values (scatterplots) with group median indicated. **(c)** Frequency distribution of DI during the test. Stacked bar charts represent the percentage of animals displaying positive or negative DI

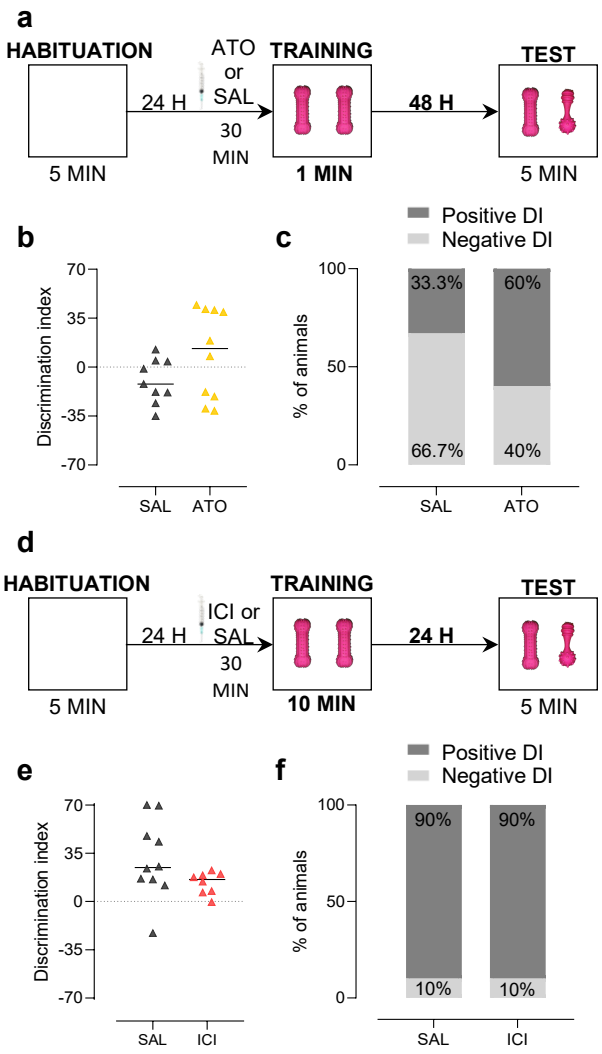


Fig. 7 Novel Object Recognition (NOR) protocols for Experiment 4 (short training, long training-test interval; males only) **(a)** and 5 (long training, short training-test interval; males only) **(d)**, with schematic timelines showing experimental phases, session durations, and inter-session intervals. **(b)** Discrimination index (DI) and **(c)** frequency distribution of DI for Experiment 4: panels show individual values (scatterplots) with group medians, as well as stacked bar charts representing the percentage of animals displaying positive or negative DI for saline (SAL) or atomoxetine (ATO) groups. Panels **(d)** and **(e)** show DI and frequency distribution of DI for Experiment 5, with respective individual values (scatterplots) and stacked bar charts for saline (SAL) or ICI-118,551 (ICI) groups

TABLE 1. Correlation between discrimination index (DI) and c-Fos expression

Brain region	Male (τ, 95% CI, p)	Female (τ, 95% CI, p)
CA1	0.07 [-0.34, 0.46], 0.65	0.07 [-0.37, 0.46], 0.7
CA3	0.19 [-0.19, 0.57], 0.29	0.09 [-0.31, 0.46], 0.62
DG	0.29 [-0.02, 0.59], 0.11	0.18 [-0.53, 0.23], 0.35
PRH	0.22 [-0.26, 0.65], 0.22	0.23 [-0.12, 0.52], 0.27

Table 1 Kendall's tau correlations between discrimination index (DI) and c-Fos expression in hippocampal subregions CA1 and CA3, dentate gyrus (DG), and perirhinal cortex (PRH) in male and female mice. For each correlation, τ values, p values, and 95% confidence intervals are reported.

TABLE 2. Correlation between discrimination index (DI) and c-Fos expression

Brain region	τ, 95% CI, p
CA1	0.05 [-0.28, 0.39], 0.74
CA3	0.13 [-0.24, 0.48], 0.39
DG	0.17 [-0.10, 0.44], 0.27
PRH	0.07 [-0.37, 0.46], 0.64

Table 2 Kendall's tau correlations between DI and c-Fos expression in hippocampal subregions CA1 and CA3, dentate gyrus (DG), and perirhinal cortex (PRH). For each correlation, τ values, p values, and 95% confidence intervals are reported.