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Neuronal correlates of spatial memory updating: c-Fos and GAD67 expression in the object-place recognition task

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The retrieval of spatial memory is behaviorally often assessed in tasks like the classical Object-Place-Recognition (OPR) task that requires an updating of memory. The neural dynamics across interconnected brain regions underlying retrieval processing in such tasks, are not well understood. Here, we examined in rats the neural correlates of retrieval processing in the OPR task by comparing a condition of successful OPR retrieval, in which the displaced object during the retrieval phase (3-hours after encoding) induced a spatial memory updating (OPR condition), with a stationary (STA) control condition in which the object configuration at retrieval was identical to encoding and, therefore, did not require spatial updating. Importantly, both conditions involved re-exposure to the previously encountered objects and context, but only the OPR condition imposed demands on updating the stored spatial representation. Expression of c-Fos (as an activity marker) and GAD67 (to identify inhibitory interneurons) was assessed at retrieval in hippocampal subfields, mPFC areas, thalamic nuclei, and posterior cortical regions. Rats in the OPR condition exhibited robust object-place discrimination and enhanced rearing behavior compared to the STA condition. Regional c-Fos activation levels were largely comparable between OPR and STA conditions across hippocampal, cortical, and thalamic regions. However, the OPR condition showed a region-specific increase in the proportion of GAD67-positive active neurons in proximal CA1. Together, these findings identify a specific pattern of inhibitory engagement implicating selective hippocampal inhibitory recruitment in supporting retrieval-driven spatial memory updating rather than elevated global activation.

Keywords Spatial memory, Object-place recognition, c-Fos, GAD67, Inhibition, Functional connectivity

Spatial memory is a fundamental ability that enables animals to navigate their environment by forming and retrieving internal representations of space. In rodents, this capacity is frequently assessed by exploiting their innate preference for novelty, typically by introducing a change into an otherwise familiar spatial configuration[1–3]. One widely used behavioral paradigm is the OPR task, in which during encoding the animal is exposed to two identical objects in an arena and at retrieval testing one of the two objects is displaced from its original location. The time the animal spends at retrieval testing with preferentially exploring the displaced object over the non-displaced object is taken as a behavioral read-out of spatial memory[1,4,5]. The animal's response to the displaced object can thus be considered a read-out of retrieval-driven spatial memory updating.

Spatial memory formation and retrieval depend on the coordinated activity of distributed neural circuits, with the hippocampus and medial prefrontal cortex (mPFC) playing central roles[6–8]. Lesions to the hippocampus consistently disrupt object-location memory across different behavioral paradigms[5,9,10]. Moreover, neuronal activation markers such as expression of the immediate early gene c-Fos have revealed robust activation of the dorsal dentate gyrus (DG) and cornu ammonis 1 and 3 (CA1 and CA3) hippocampal subregions during OPR retrieval[11]. Importantly, c-Fos expression in dorsal CA1 was positively correlated with successful OPR retrieval[11]. Within the dorsal CA1 region, a functional gradient along the hippocampal proximal-distal axis

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has been proposed, whereby spatial information is mainly encoded into proximal CA1 (pCA1), adjacent to CA2, while the distal CA1 (dCA1) is thought to be more involved in processing object-related information[12,13]. However, other studies have reported that pCA1 and dCA1 exhibit comparable spatial selectivity[14]. Moreover, there may be a complementary gradient along the dorsoventral axis of CA1[15].

The mPFC, particularly the prelimbic (PL) and infralimbic (IL) cortices, plays a significant role in spatial memory through both direct and indirect interactions with the hippocampus[16–18]. Lesions to the IL and PL in rats have been shown to impair the acquisition and retrieval of OPR and other spatial-cue dependent tasks[17–19], and neurons in these regions exhibit location-specific firing patterns during spatial navigation[20]. Projections from the mPFC to the hippocampus modulate behavioral expression of memory and are necessary for successful retrieval of object-in-context memory[7,8,16,21,22]. The pathways mediating mPFC-hippocampal interactions comprise, among others, the thalamic nucleus reuniens (RE)[23–25], as well as the perirhinal cortex (PRH) and lateral entorhinal cortex (LEC), which appear to support a mPFC-mediated top-down control during the retrieval of context-appropriate memory representations in the dorsal hippocampus[6,26,27].

Recent evidence points to a critical role of hippocampal inhibitory interneurons in spatial memory and the emergence of the cognitive map[28], although interneurons comprise only 10–15% of the total neuronal population in the hippocampus[29]. A monosynaptic inhibitory projection from the prefrontal cortex to dorsal hippocampus has been shown to facilitate object-related exploration and encoding through top-down control[30]. Also, local inhibitory circuits within the hippocampus appear to play an important role in spatial learning and memory[31,32].

Here, we aimed at dissociating the networks mediating retrieval-driven memory updating from mere retrieval processing by comparing activity during successful retrieval in a standard OPR task with a stationary control condition in which the object configuration at retrieval remained the same as during encoding and thus did not require spatial updating. During the retrieval phase, the expression of c-Fos and GAD67 was measured to discriminate activity of excitatory and inhibitory neurons, with the analyses targeting mainly hippocampal and prefrontal subregions. Rather than observing increases in excitatory activity within individual areas, we found that retrieval-driven memory updating during the OPR test was associated with a selective increase in inhibitory activity in CA1.

Results

In order to characterize retrieval and updating of spatial memory, behavioral performance and neuronal activity during the retrieval phase of a standard OPR task (OPR condition) were compared with that in a stationary (STA) control condition in which object positions remained unchanged between encoding and retrieval (Fig. 1A). In the STA condition, the absence of spatial reconfiguration eliminated the need for retrieval-driven spatial updating while maintaining task exposure.

Behavioral indices of spatial memory retrieval

Evaluation of object exploration times for the two objects during the retrieval phase confirmed successful memory retrieval, indicated by the presence of significant object-place recognition memory exclusively in the OPR condition. Only this condition showed a preference for exploring the displaced over the stationary object (one-sample t-test: $t(11) = 3.00$, $p = 0.012$; $t(11) = 4.94$, $p < 0.001$, for 1 min and at 3 min, respectively). As expected, no such preference was observed in the STA condition (all $p > 0.192$). Accordingly, the discrimination index of retrieval performance was also significantly higher in the OPR condition than in the STA condition ($F(1, 11) = 6.48$, $p = 0.027$, for ANOVA main effect of Group; pairwise comparisons: $p = 0.049$ at 1 min and $p = 0.035$ at 3 min) (Fig. 1B). Importantly, the effect of Group did not interact with task order ($F(4,20) = 0.1487$, $p = 0.96$, for respective Group x Order interaction), excluding that performance on the OPR task, when occurring on the animal's second session, was in any way substantially biased by prior task experience on the first session (Figure S1). Moreover, control variables did not differ between conditions (Fig. 1C-D). Total object exploration time during encoding (paired samples t-test: $t(11) = -1.225$, $p = 0.246$) and Total locomotor activity (distance travelled) during the 3-min retrieval phase (paired samples t-test: $t(11) = -0.940$, $p = 0.367$) was the same across conditions.

We additionally assessed rearing behavior (Rearing duration, Rearing number, Mean rearing duration) during retrieval, as another exploratory behavior that typically increases in response to changes experienced in the spatial environment[33,34](Fig. 1E-G). Consistent with this concept, the OPR condition, exposed to the configurational change in the two objects, showed significantly longer rearing duration than the STA condition, particularly within the first three minutes of the retrieval phase ($F(1, 11) = 4.567$, $p = 0.056$ and $F(1,11) = 7.481$, $p = 0.019$, for ANOVA Group x Time interaction and Group main effect, respectively; pairwise comparisons: $p = 0.226$ at 1 min, $p = 0.019$ at 3 min). However, no significant group differences were observed for Rearing number ($F(1,11) = 0.015$, $p = 0.906$ for the Group × Time interaction; $F(1,11) = 2.602$, $p = 0.135$ for the Group main effect) or for Mean rearing duration ($F(1,11) = 3.426$, $p = 0.091$ for the Group × Time interaction; $F(1,11) = 0.746$, $p = 0.406$ for the Group main effect).

Overall, these results indicate that successful object-place recognition under spatial updating demands, as observed in the OPR condition, was characterized by selective exploration of the displaced object and increased rearing behavior during the retrieval phase.

c-Fos activation

Analysis of total c-Fos in the 16 target brain regions revealed patterns of activity across regions that did not significantly differ between the OPR and STA conditions ($F(15, 105) = 0.931$, $p = 0.53$, Group x Area interaction in a global ANOVA across all 16 areas, Fig. 2A-B). c-Fos activity in excitatory cells, i.e., c-Fos+ GAD67- cells, followed the same pattern ($F(15, 105) = 0.902$, $p = 0.564$, Group x Area interaction, Figure S2). Focusing on the hippocampal subregions (vCA1, dCA1, pCA1, CA3 and DG, Fig. 2B), we examined whether regional activation

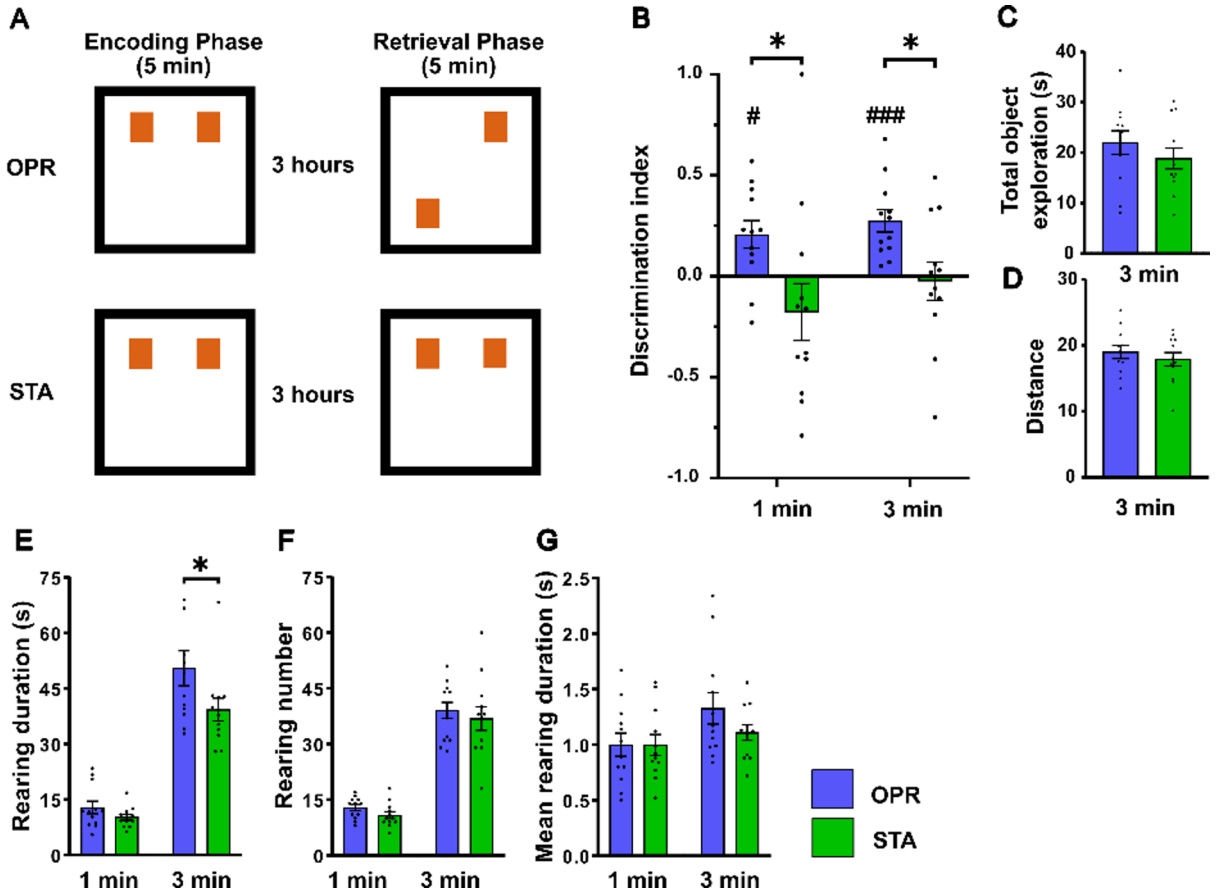

Fig. 1. OPR performance. **(A)** Experimental design. Following a within-subject design, adult rats were tested in the standard OPR task and in a stationary control condition (STA), in which the two objects remained in the same location during both encoding and retrieval phases. Ninety minutes after retrieval, brains from both conditions were collected for c-Fos and GAD67 immunohistochemistry. **(B)** Object discrimination index for the first and first three minutes of the retrieval phase. # $p < 0.05$, ### $p < 0.001$ for one sample t-test against 0. **(C)** Total object exploration for the first three minutes of encoding. **(D)** Distance traveled during the first three minutes of retrieval. **(E)** Rearing number, **(F)** Rearing duration and **(G)** Mean rearing duration for the first and first three minutes of the retrieval phase. * $p < 0.05$, ANOVA with post hoc multiple comparisons. Data are presented as mean ± SEM with individuals subjects values overlaid.

differed between OPR and STA conditions. No significant main effect of Group or Group × Area interaction was observed across hippocampal subregions ($F(1, 10) = 2.991$, $p = 0.114$, for Group and $F(4, 40) = 1.478$, $p = 0.227$ for Group x Area interaction), indicating comparable levels of c-Fos expression during retrieval in both conditions (Fig. 2B). c-Fos activation patterns across mPFC subregions (PL, IL, CG, Fig. 2B) and posterior cortical regions (LEC, PRH, RSG, RSD, PAR; Fig. 2B) did not differ significantly between OPR and STA conditions (mPFC: $F(2, 20) = 1.663$, $p = 0.215$, posterior cortex: $F(4, 32) = 1.432$, $p = 0.246$, for Group x Area interaction). Likewise, thalamic areas (RE and RTN) showed no differences between conditions ($F(1, 10) = 1.534$, $p = 0.224$, for Group x Area interaction).

Overall, these findings indicate that retrieval-driven updating of spatial memory was not associated with increased regional c-Fos activation magnitude compared to the stationary control condition.

Inhibitory modulation

To assess inhibitory contributions to spatial memory retrieval, we examined the proportion of the total number of active neurons expressing c-Fos that co-expressed GAD67 across the 16 brain areas (Fig. 3A-B). A global analysis on all regions indicated that inhibitory recruitment did not initially differ across groups ($F(15, 105) = 1.680$, $p = 0.066$ for Group × Area interaction). However, group specific effects were revealed in the proportion of active GAD67 + neurons on hippocampal subregions ($F(4, 40) = 5.040$, $p = 0.002$ for Group × Area interaction). Notably, in the OPR group the percentage of GAD67 + active neurons in the pCA1 region was distinctly higher than in the STA ($p = 0.013$), suggesting a targeted inhibitory recruitment during successful retrieval. Such an effect was not observed in the dCA1 ($p = 0.684$), supporting a functional dissociation along the proximal-distal axis (Fig. 3B). In other hippocampal regions (vCA1, CA3, DG) inhibitory activity was comparable between groups (all $p \geq 0.109$).

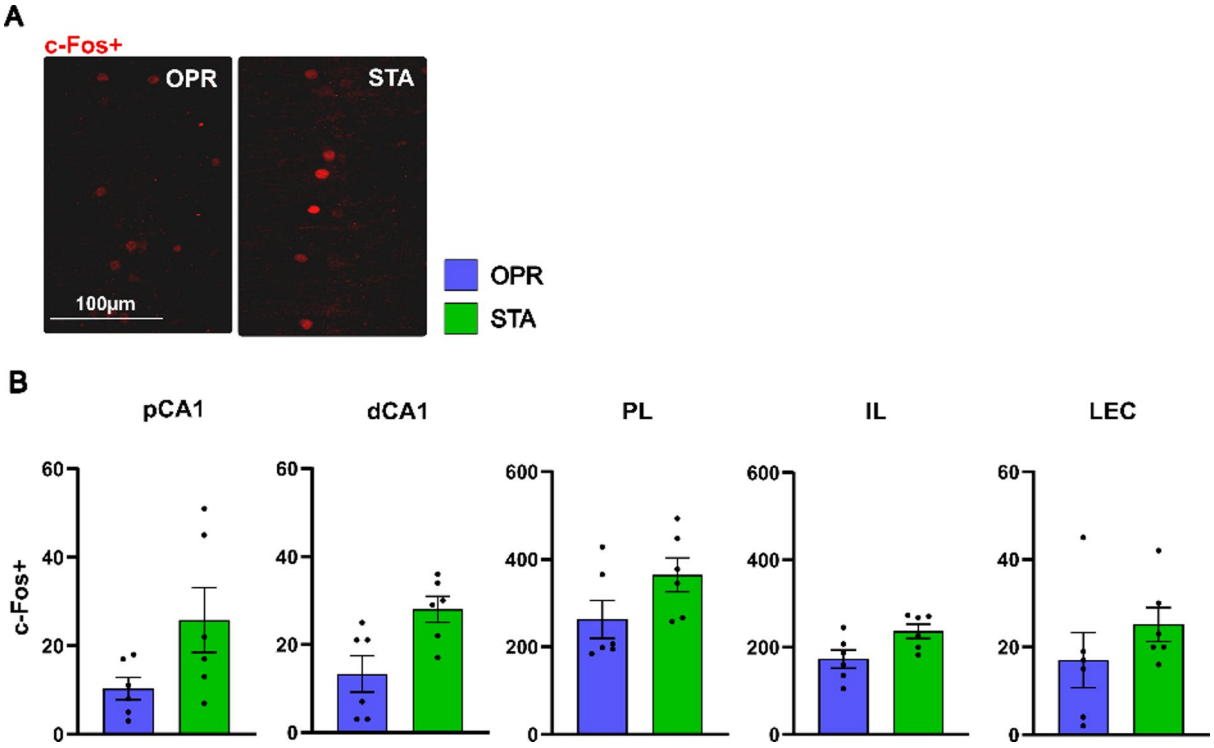

Fig. 2. c-Fos expression during retrieval. **(A)** Confocal images showing c-Fos expression in part of dCA1 in OPR and STA **(B)** Total c-Fos+ cell counts in pCA1, dCA1, PL, IL and LEC. * $p < 0.05$, RM-ANOVA with post hoc multiple comparisons. Data are presented as mean ± SEM with individual subject values overlaid.

The proportion of GAD67 + active cells in the mPFC did not differ among groups ($F(2, 20) = 1.633$, $p = 0.220$ for Group × Area interaction), and no clear group differences in the proportion of GAD67 + active neurons were found in thalamic ($F(1, 10) = 0.007$, $p = 0.935$ for Group × Area interaction) or posterior cortical regions ($F(4, 32) = 0.799$, $p = 0.535$ for Group × Area interaction).

In complementary analyses, we also assessed the number of GAD67 + neurons co-labelled with c-Fos, which generally revealed patterns similar to those from the analysis of c-Fos+ cells ($F(15, 105) = 1.127$, $p = 0.342$, for Group x Area interactions, Fig. 3C).

Taken together, these findings indicate that spatial memory retrieval under spatial updating was characterized by a localized increase in the proportion of active inhibitory neurons in proximal CA1, whereas inhibitory recruitment in other regions remained comparable to the stationary control condition.

Brain functional connectivity

In an exploratory analysis, we assessed functional connectivity in the network of the 16 targeted brain regions based on examining correlations in c-Fos expression during retrieval between these regions. Pairwise Pearson correlation coefficients for activity between the regions were calculated for each group, and coefficients of $r > |0.706|$ (corresponding to an uncorrected $p < 0.05$) were considered to reflect above-threshold connectivity between the respective regions (Fig. 4). The total number of above-threshold interregional correlations in the OPR group showed a trend-level increase compared to the STA group ($\chi^2 = 3.559$, $z = 1.887$, $p = 0.059$). All above-threshold correlations in the OPR group were positive, indicating coordinated activation rather than antagonistic dynamics.

Discussion

This study aimed to clarify the neuronal correlates underlying the behavioral expression of effective retrieval of spatial memory in a standard OPR task. Retrieval behavior and associated brain c-Fos activity during standard retrieval conditions were compared with a STA control condition in which the object configuration at retrieval was identical to encoding and, therefore, did not require spatial updating. Importantly, both conditions involved re-exposure to the previously encountered objects and context, but only the OPR condition imposed demands on updating the stored spatial representation due to displacement of one object.

We revealed a specific behavioral and c-Fos-related profile for the OPR group that contrasted with the STA control condition. Thus, the OPR group showed, at the behavioral level, clear preferential exploration of the displaced object during the retrieval phase (i.e., a significant discrimination index), and enhanced level of rearing behavior compared to the STA condition. Meanwhile, c-Fos activity in this condition was largely comparable to those observed in the STA condition across most regions. Finally, there was a local increase in inhibition in the proximal CA1 region that was characteristic of the OPR group compared to the STA condition.

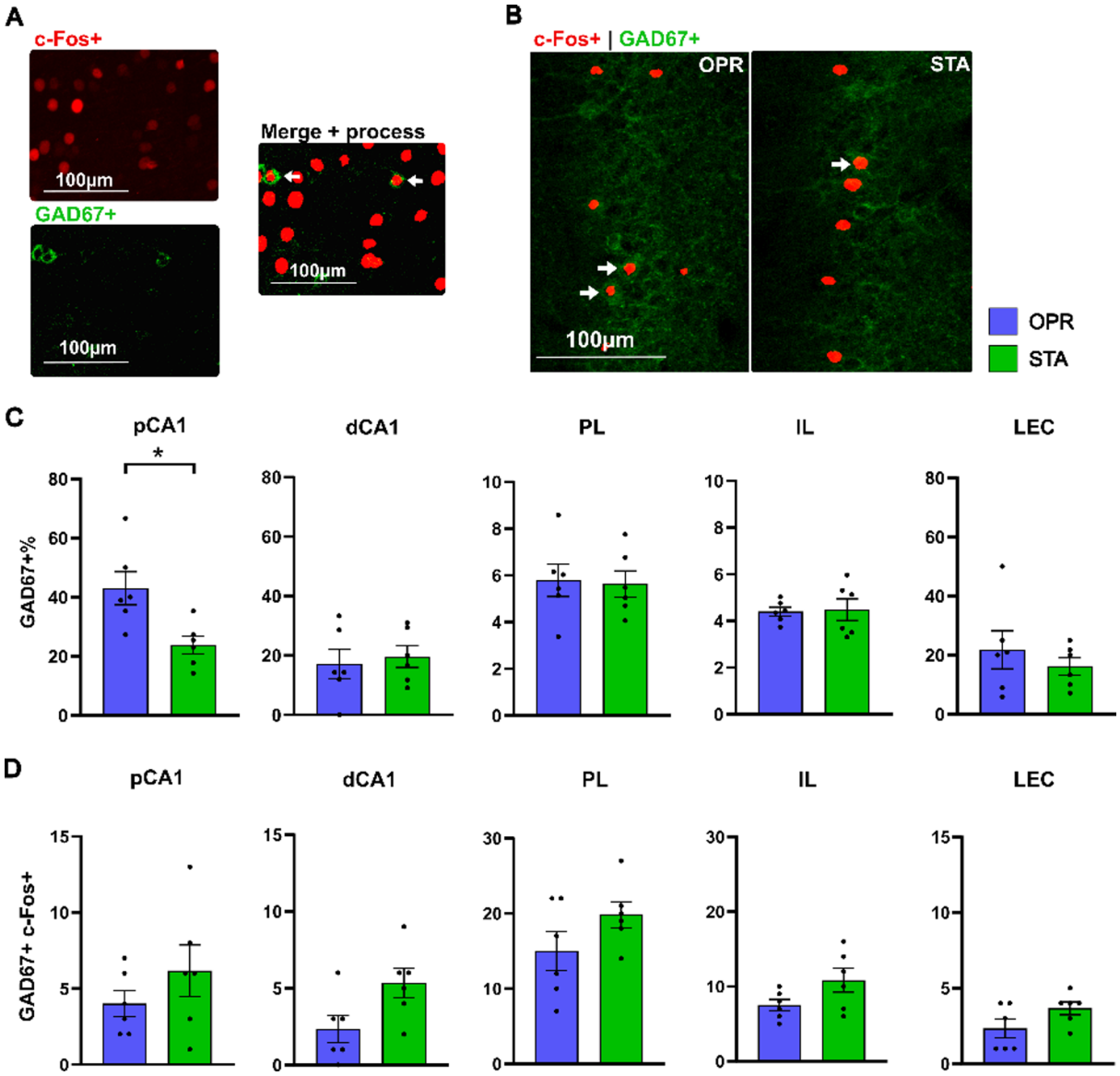

Fig. 3. GAD67 expression during retrieval. **(A)** Representative confocal images. Top left panel shows c-Fos+ stained cells, bottom left panel shows GAD67 + stained cells and the right panel the merged figure after applying threshold for c-Fos in CellProfiler. Arrows point to co-expression of GAD67 and c-Fos. **(B)** Confocal images showing c-Fos and GAD67 expression in part of dCA1 in OPR and STA. Arrows point to co-expression of GAD67 and c-Fos. **(C)** Percentage of GAD67 + c-Fos+ cells (with total number of cFos+ cells set to 100%) in pCA1, dCA1, PL and IL. **(D)** Total numbers of GAD67 + c-Fos+ cells in pCA1, dCA1, PL, IL and LEC. * $p < 0.05$, RM-ANOVA with post hoc multiple comparisons. Data are presented as mean ± SEM with individual subject values overlaid.

Our behavioral results confirm that rats in the OPR group successfully retrieved the memory. In conjunction with the STA group showing no preferential object-exploration, the findings validate the use of object-exploration in this paradigm as an indicator of spatial memory retrieval[2–5,35]. The differences between groups in rearing behavior were complementary to their differences in object exploration. Exploratory rearing is considered a marker of spatial memory updating based on distal environmental cues[33,34,36–38]. Accordingly, the increased rearing duration in the OPR group compared to the STA group likely reflects a response to the reconfigured spatial environment requiring updating of the previously encoded representation. In the STA condition, retrieval of object information occurred without the need to modify the stored spatial configuration, and correspondingly rearing remained lower. Taken together, the behavioral profile of enhanced preferential object exploration and rearing in the OPR group compared to the stationary control condition can be considered to reflect successful retrieval accompanied specifically by spatial updating demands[36–38].

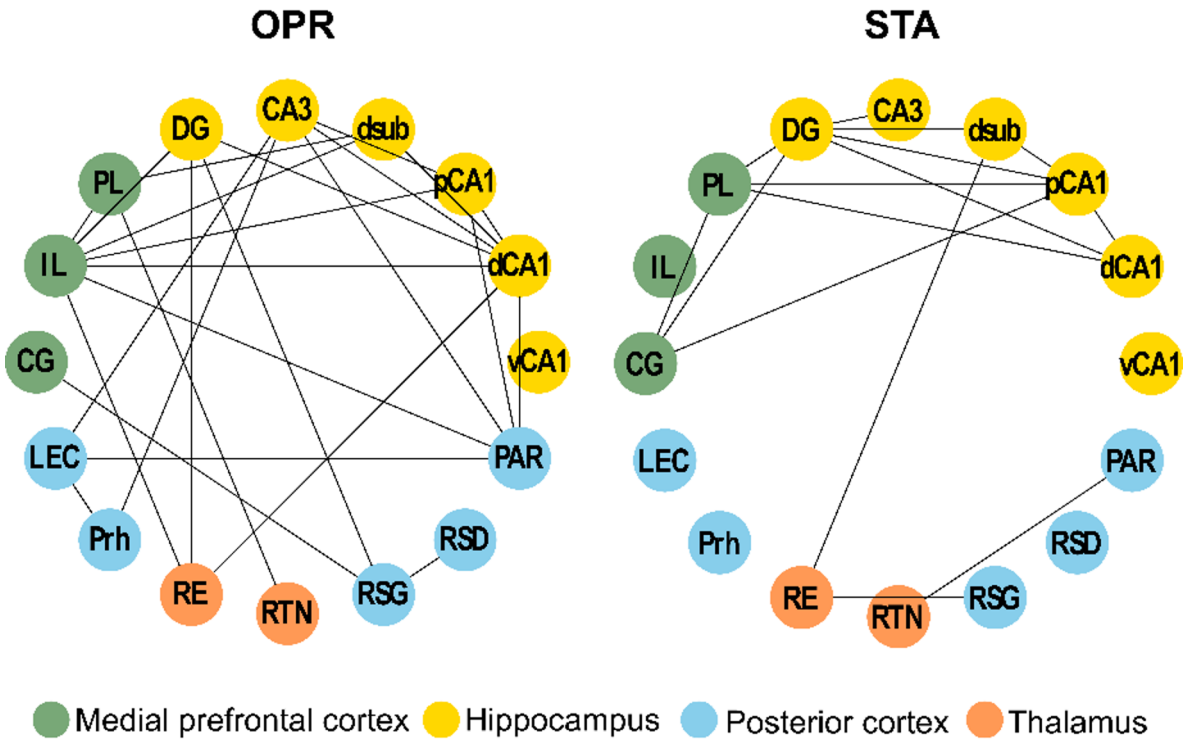

Fig. 4. Functional connectivity based on correlated c-Fos activity between brain regions. Network graphs from OPR and STA conditions. Yellow circles depict hippocampal subregions, green circles represent mPFC subregions, blue circles indicate posterior cortex subregions and in orange, the thalamic subregions. Connecting lines represent statistically significant correlations ($\chi^2 = 3.559$, $z = 1.887$, $p = 0.059$).

c-Fos is a widely used non-specific marker of neuronal activity[39]. In this context, c-Fos expression in the OPR group likely reflects neuronal processing associated with the retrieval of the stationary object and arena context, alongside the encoding of the novel (displaced) object location.

Importantly, regional c-Fos levels during OPR retrieval did not differ from those observed in the STA condition across hippocampal, cortical and thalamic areas. This indicates that retrieval per se, when not accompanied by spatial updating demands, was associated with comparable regional activation levels. However, these findings differ from previous reports describing enhanced c-Fos expression during OPR retrieval in hippocampal and other regions[11,40]. The reasons for this discrepancy are not entirely clear but may relate to differences in control conditions or experimental design. Compared with the study by Mendez et al. (2015)[11] which also employed a Stationary control, the animals of the present study – in the context of our within-subject design – might have been less habituated to the objects but more habituated to contextual aspects of the arena[41]. Nevertheless, exploratory analyses indicated that OPR performance did not differ between animals performing the task first or second (Figure S1), arguing against a substantial influence of prior task exposure on behavior or associated neuronal activation during retrieval. Taken together, these findings suggest that c-Fos expression during the OPR retrieval phase may partly reflect subtle differences in encoding history or task experience. Alternatively, they may indicate that local c-Fos levels are not sufficiently sensitive to detect subtle distinctions in spatial retrieval processing within hippocampal regions. This conclusion likewise aligns with our failure to find differences in c-Fos expression between the OPR and STA conditions along the proximal-distal axis of the CA1[12,13,42].

There were, however, distinct local changes in c-Fos expression in the OPR group revealed by the analyses focusing on GAD67 + inhibitory interneurons. Retrieval-driven memory updating in the OPR group was associated with a localized increase in active GAD67 + neurons specifically in the pCA1, compared with the STA condition. This localized inhibition aligns with evidence indicating that reduced GAD67 expression in the dorsal hippocampus is linked to impaired spatial performance[43]. A wealth of electrophysiological findings also show that hippocampal interneurons, through feedforward and feedback inhibition sharpen excitatory input integration, thereby enhancing signal-to-noise ratios, and refining the respective spatial representations through selective neuronal recruitment[29,44–46]. In this context, the increased proportion of active GAD67 + cells in the pCA1 region of our OPR group may well reflect a circuit-level mechanism that ensures precision in memory updating by suppressing irrelevant or competing signals.

A growing body of research shows that brain networks supporting episodic memory processing differ based on memory content and, thus, can be inferred from correlated c-Fos expression across the relevant regions[40,47]. In line with this work, exploratory functional connectivity analyses revealed a tendency toward a greater number of significant interregional correlations in the OPR condition compared to the stationary control condition. However, this difference did not reach statistical significance and should therefore be interpreted with caution.

Although not definitive, this trend is consistent with the possibility that successful retrieval requiring spatial updating may involve more coordinated engagement of distributed hippocampal–prefrontal–thalamic circuitry. Further studies with greater statistical power or complementary methodologies will be necessary to determine whether such network-level differences reliably characterize retrieval-driven updating processes.

This study aimed to dissect the neural dynamics underlying retrieval-driven spatial memory updating in the standard OPR task by integrating behavioral performance with the expression of c-Fos and GAD67 as well as exploratory interregional functional connectivity analyses. Overall, the findings indicate that retrieval requiring spatial updating was not associated with heightened regional neuronal activation per se, but was characterized by a selective increase in inhibitory recruitment in proximal CA1, accompanied by a trend toward greater interregional coordination. While these results provide novel insight into the neuronal mechanisms supporting retrieval processing under updating demands, several limitations restrict the conclusiveness of our findings. Most importantly, the temporal resolution of c-Fos expression is limited, preventing precise allocation of activity changes to specific cognitive subprocesses, and it remains difficult to dissociate these processes based solely on behavioral measures. Additionally, although the STA condition effectively controlled for nonspecific influences and allowed to dissociate retrieval from spatial updating processes, the absence of a classical home-cage control limits the extent to which task-specific activation can be fully separated from generalized arousal effects. Taken together, these considerations highlight the need for future studies that further delineate the circuit mechanisms underlying spatial memory retrieval with and without updating demands. Pharmacological or optogenetic manipulations may provide causal evidence linking selective hippocampal inhibitory recruitment -and potentially network-level coordination- to behavioral retrieval performance.

Materials and methods

Animals

A total of 12 male Long-Evans rats were used in the experiments. Data was derived from previously published data (Experiment 2 in[38]. All animals were obtained from Janvier Labs (Le Genest-Saint-Isle, France) and arrived at our facility at least five days prior to any experimental procedure to allow for acclimatization. Rats arrived at 9 or 10 weeks of age and were housed in pairs. The animal colony was maintained under standard conditions: a constant room temperature of 22 ± 1 °C and a controlled 12/12 h light/dark cycle (lights on at 07:00). Food and water were available *ad libitum* throughout the experimental period. All procedures were performed in accordance with the European animal protection laws and were approved by the Baden-Württemberg state authority (Regierungspräsidium, Tübingen, Baden-Württemberg, Germany).

Behavioral procedures

The two experimental groups (PD80-94) were designed to examine how retrieval-related neural activity varies under different spatial conditions in the OPR task. The groups followed a within-subjects design, i.e., on the day following the last habituation session, six rats were tested on the standard OPR task, and after a one-week interval, the same rats performed the STA task.

In the OPR group, one of the two identical objects was displaced during retrieval, introducing a minimal degree of spatial novelty typically associated with successful memory expression (Fig. 1A). In the STA group, object positions remained unchanged between encoding and retrieval, serving as a control for exploration and task exposure without spatial reconfiguration.

All experiments were performed during the light phase, between 7:00–14:00 h. Prior to spatial memory testing, all rats underwent daily handling sessions (5–10 min per day) for three to five consecutive days. Handling was followed by three days of habituation, during which animals freely explored an empty open field for 10 min per session, being introduced from different sides each day. Following these sessions, the rats were further habituated in pairs to a resting box for a 6-hour period. The day after the final habituation session, rats underwent the OPR task. On the task, two identical objects were positioned 9 cm and 15 cm from the respective smaller and larger open field walls. The encoding phase allowed a 5-minute exploration interval. During the subsequent 3-hour retention interval, the rats remained (in pairs) undisturbed in the resting box. In the retrieval phase, one of the two objects from the encoding phase was relocated while the other remained in its original position. The retrieval phase allowed the animal to explore the objects for 5 min. Each rat entered the arena from a different side in both encoding and retrieval phases to prevent that entrance position is used as a proximal cue. Object locations were counterbalanced across rats. The STA group followed the same procedures as the OPR task, except that during the retrieval phase, both objects remained in their original locations.

Apparatus

All behavioral tasks were conducted using a square open field arena made of dark grey PVC with a 77 × 77 cm arena and 37 cm-high walls. The arena had a North side facing a white wall, while the East and West sides were flanked by grey curtains. A removable black curtain marked the South side and served as the experimenter's entrance. Distal cues were placed on the ceiling above the arena: a 40 × 40 cm wooden square located 120 cm above the floor and 36 cm below the ceiling. At its center, a 5 × 7 cm metal square served as both a visual cue and support for the overhead video camera. Two additional cues were attached to the surrounding curtains: a pink ball (10 cm diameter) and a light-brown cardboard box (25 × 25 × 10 cm).

Objects used in the task were glass bottles of various shapes (22–29 cm tall), filled with sand to provide sufficient weight and prevent displacement by the animals. Indirect lighting was provided by fluorescent strips mounted beside and beneath the arena. A constant white noise was played throughout all procedures to mask ambient sounds. After each session, the arena and objects were cleaned thoroughly with a 70% ethanol solution.

Analysis of memory performance

Animal behavior was recorded using a video camera positioned above the center of the open field. Rearing behavior and object exploration were visually scored using ANY-Maze tracking software (Stoelting Europe, Dublin, Ireland), and quantified for each session, as previously described[33]. Object exploration was defined as the rat being within 2 cm of an object, with its nose directed towards the object and actively engaging in behaviors such as sniffing. Behaviors like sitting, standing, or rearing next to the object, without active exploration, were not considered exploratory. Memory retrieval was assessed using the object discrimination index (DI), a standard measure of OPR performance. The DI was calculated as follows: DI = (exploration time for the novel object-location – exploration time for the familiar object-location) / (exploration time for the novel object-location + exploration time for the familiar object-location). A positive DI, indicating a preference for the displaced object, is considered as evidence of successful memory retrieval of the stationary (familiar) object. Accordingly, a DI of zero, indicating no preference, is considered to reflect a failure to recall the original configuration (i.e., the familiar object). For the STA group, object identities were arbitrarily designated as "novel object location" or "familiar object location" for the purpose of calculation, as no objects were actually displaced and, consequently, an expression of retrieval through object preference is not anticipated. In addition, total object exploration time (across both objects) and total distance travelled (normalized) during retrieval phases were measured to indicate motivation and locomotion.

An exploratory rearing event was defined when the rat stood on its hind legs, lifting its forelegs off the ground, with or without support from the arena wall. Rearing was only scored if the animal raised its head high and appeared to be scanning its environment. For both encoding and retrieval phases, the number of rearing events (Rearing number), total rearing duration (Rearing duration), and mean rearing duration (Mean rearing duration; total rearing duration divided by the number of rearing events) were calculated.

c-Fos and GAD67 immunocytochemistry

After completing the retrieval phase of the task, all rats were returned to their home cages for 90 min. Subsequently, they were euthanized by transcardial perfusion under deep anesthesia induced with a mixture of Midazolam (4.0 mg/kg), Medetomidin (0.3 mg/kg), and Fentanyl (0.01 mg/kg) administered intraperitoneally. Perfusion was performed first with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were post-fixed for 24 h at 4 ◦C and stored in PBS at 4 ◦C until further processing (OPR: $n = 6$; STA: $n = 6$). Coronal slices (70 µm) at the respective bregma levels were cut with a vibratome (Microm HM 650 V, Thermo Fisher Scientific MICROM GmbH, Germany). For immunostaining, free-floating sections were blocked with PBS containing 10% horse serum and 0.3% Triton-X for 90 min. Afterwards, the sections were incubated with 0.2% Triton-X in PBS containing mouse monoclonal anti-GAD67 (1:1,000 dilution; Sigma, #MAB5406) and rabbit polyclonal anti-c-Fos (1:1,000 dilution; Synaptic Systems, #226 003) primary antibodies for 48 h at 4 °C. The sections were washed with PBS four times and, then, incubated with 0.2% Triton-X in PBS containing donkey anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 (1:1,000 dilution; Thermo Fisher Scientific) secondary antibodies for 24 h at 4 °C. Samples were incubated with NeuroTrace (1:200 dilution; Thermo Fisher Scientific), washed with PBS, mounted on gel-coated slides, and coverslipped with Vectashield anti-fade mounting medium (Vector Laboratories, USA).

The regions of interest (ROIs) were selected based on previous literature implicating them in spatial memory formation and were anatomically defined according to the atlas by Paxinos and Watson (2006)[48]. ROIs and their distance (in mm) from Bregma were as follow: +3.24 mm for prelimbic (PL), infralimbic (IL), and cingulate cortex (CG); -1.8 mm for thalamic reuniens (RE) and thalamic reticular nucleus (RTN); -3.96 mm for proximal CA1 (pCA1), distal CA1 (dCA1), CA3, DG, retrosplenial cortex granular (RSG), retrosplenial cortex dysgranular (RSD), parietal (PAR), perirhinal cortex (PRH) and lateral entorhinal cortex (LEC); -5.76 mm for ventral CA1 (vCA1), dorsal subiculum (dSub), ventral subiculum (vSub); -7.68 mm for medial entorhinal cortex (MEC) (Figure S3).

Images were obtained using a confocal laser scanning microscope (LSM 710; Carl Zeiss, Germany) with a low magnification (plan-apochromat oil-immersion objective, 25×/0.8NA; z- stack of 11 slices (50 µm thickness); voxel size 0.33 µm × 0.33 µm × 3.5 µm (Figs. 2A and 3A-B). Settings for laser intensity, gain, offset and pinhole were optimized initially and held constant throughout the experiments. The number of cells expressing c-Fos and GAD67 were counted automatically (for c-Fos+ cells) and manually (for GAD67 + cells) using CellProfiler software. Counting was done on the maximum intensity of the z-stacks of the entire slice volume. The sizes of the ROIs were 809,200 μm^2 for PL, 462,400 μm^2 for IL, 578,000 μm^2 for CG, 462,400 μm^2 for RE, 346,800 μm^2 for RTN, 231,200 μm^2 for pCA1, 346,800 μm^2 for dCA1, 462,400 μm^2 for CA3, 289,000 μm^2 for DG, 346,800 μm^2 for RSG, 346,800 μm^2 for RSD, 404,600 μm^2 for PAR, 346,800 μm^2 for PRH, 173,400 μm^2 for LEC, 346,800 μm^2 for vCA1, 323,000 μm^2 for dSub, 221,952 μm^2 for vSub and 173,400 μm^2 for MEC. All analyses followed previously reported standard procedures[34,49] and were done by an experimenter blind to the individual rat's experimental condition.

Statistical analysis

Statistical comparisons were performed using SPSS software (IBM, Armonk, NY, USA). Behavioral exploration was quantified for both exploration during the first minute (1 min) and during the first three minutes (3 min) of the retrieval phase. The analyses were restricted to the first 3 min of the retrieval phase because this initial interval has been consistently found to most sensitively reflect behavioral memory effects of the task in adult rats whereas exploratory behavior declines thereafter making longer bins, such as 5-minute intervals, less informative[5,50–53]. To compare OPR and STA behavior, a 2 × 2 repeated measures ANOVA was used, with Time bin (1 min/3 min) and Tasks (OPR/STA) as repeated measures. Moreover, we added an Order (1st/2nd) factor to these ANOVA to examine whether behavioral effects on the tasks depended on the order in which the animals

encountered the OPR and STA tasks. Significant ANOVA main or interaction effects were followed by post-hoc tests. One sample t-test was used to test whether discrimination ratio significantly differed from zero. A one-way ANOVA was conducted to compare the total distance traveled and total object exploration during the 3-minute retrieval phase between groups.

For the c-Fos analyses, minor tissue damage in some sections led to incomplete datasets, and as a result, some ROIs did not include all animals from each group in the statistical analyses (vSub: STA, $n = 5$; OPR, $n = 4$; MEC: STA, $n = 3$; OPR, $n = 3$; PAR: STA, $n = 5$; RSD: STA, $n = 4$; RSG: STA, $n = 5$). The numbers of c-Fos+, GAD67 + c-Fos+ (inhibitory cells), percentage of GAD67+ (c-Fos+ GAD67+/c-Fos+), and the number of GAD67- c-Fos+ cells (presumably excitatory and glial cells) were first analyzed using a global ANOVA, which included a Group factor (OPR/STA) and a repeated-measures Area factor (excluding MEC and vSub due to low n), thereby reflecting the respective regions of interest. Given our particularly interest in differential activation of subregions within the cortical, thalamic, and hippocampal target structures, separate ANOVAs were performed for the mPFC (PL, IL, and CG), the hippocampus (vCA1, dCA1, pCA1, CA3 and DG), the posterior cortex (LEC, PRH, RSG, RSD and PAR) and the thalamus (including RE, RTN), followed by a post-hoc tests with Sidak correction in cases of significant effects.

For the exploratory functional connectivity analysis based on c-Fos activity, Pearson correlation coefficients were calculated between all pairs of brain areas of interest. Significant r values varied according to the sample size of each comparison. The total number of significant correlations was compared between groups using a χ^2 test. MEC and vSub were also excluded from this analysis due to the lower sample sizes. Connectivity graphs were constructed based on c-Fos quantifications and correlation coefficients with the Igraph package (v1.2.4.2) in R (RStudio, Boston, MA, USA).

All results are presented as means ± SEM. If not otherwise indicated, a two-sided level of $p < 0.05$ was considered significant.

Data availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
M.I. and J.B. designed the experiment, R.P., X.S. and S.D. performed the experiments and analyzed the data; R.P. drafted the manuscript; M.I., J.B. and S.D. edited and reviewed the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Animal research guidelines
This study is reported in accordance with ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

Additional information

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