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Targeting Nociceptin/Orphanin FQ receptor to rescue cognitive symptoms in a mouse neuroendocrine model of chronic stress

Running title: N/OFQ peptide receptor and stress-induced memory deficits

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Abstract

Chronic stress causes cognitive deficits, such as impairments in episodic-like hippocampus-dependent memory. Stress regulates an opioid-related neuropeptide named Nociceptin/OrphaninFQ (N/OFQ), the ligand of the G protein-coupled receptor NOP. Since this peptide has deleterious effects on memory, we hypothesized that the N/OFQ system could be a mediator of the negative effects of stress on memory. Chronic stress was mimicked by chronic exposure to corticosterone (CORT). The NOP receptor was either acutely blocked using selective antagonists, or knocked-down specifically in the hippocampus using genetic tools. Long-term memory was assessed in the object recognition (OR) and object location (OL) paradigms. Acute injection of NOP antagonists before learning had a negative impact on memory in naive mice whereas it restored memory performances in the chronic stress model. This rescue was associated with a normalization of neuronal cell activity in the CA3 part of the hippocampus. Chronic CORT induced an upregulation of the N/OFQ precursor in the hippocampus. Knock-down of the NOP receptor in the CA3/Dentate Gyrus region prevented memory deficits in the CORT model. These data demonstrate that blocking the N/OFQ system can be beneficial for long-term memory in a neuroendocrine model of chronic stress. We therefore suggest that NOP antagonists could be useful for the treatment of memory deficits in stress-related disorders.

Introduction

Chronic stress exposure in humans has an impact on cognition, notably on episodic memory, underpinned in particular by the continuous activation of the hypothalamic-pituitary-adrenal (HPA) axis [1]. In aged humans, prolonged cortisol elevations are associated with reduced hippocampal volume and deficits in hippocampus-dependent memory [2]. Such neuroanatomical and behavioral alterations are also found in rodents exposed to chronic stress or elevated levels of corticosterone (CORT) [3–7]. Yet, the mechanisms by which chronic stress induces memory deficits are not fully understood. Moreover, cognitive symptoms are also associated with major depressive disorder for which chronic stress is a known etiological factor [8]. However, there is still a lack of treatments specifically targeting cognitive impairments in depressive patients [9].

Nociceptin/Orphanin FQ (N/OFQ) is an opioid-related neuropeptide that acts through a single inhibitory G protein-coupled receptor called Nociceptin Opioid Peptide (NOP) receptor [10, 11]. This system is widely expressed in the central nervous system [12] and is linked to major physiological functions such as pain [13, 14], feeding behavior [15, 16], motivation and reward [17–20], sleep [21], and stress adaptation [22, 23]. Thus, the N/OFQ system represents an original and promising pharmacological target for numerous neurological and psychiatric diseases [24, 25], particularly stress-related pathologies [26–29].

NOP receptors and N/OFQ are particularly abundant in limbic regions and brain areas related to stress regulation in rodents and humans [30–33]. Accordingly, studies have associated the N/OFQ system with both the memory processes [34] and the stress response [29]. The administration of NOP agonists has been shown to impair spatial [35, 36], contextual aversive [37–40], and recognition [41] long-term memory. Interestingly, these amnesic effects are also observed after intrahippocampal injection

of NOP agonists [36, 41]. However, while those studies demonstrate that exogenous pharmacological activation of NOP receptor signaling is sufficient to impair memory formation and maintenance, the involvement of the endogenous N/OFQ in memory regulation associated with chronic stress and other pathological states remains elusive. Investigating how stress selectively modulates endogenous systems represents a critical and mandatory step in better understanding and treating memory deficits in stressed and older human population. In this line of thoughts, stressful conditions in rodents such as restraint [42, 43], social defeat [44], crowding [45], and traumatic stress [46] significantly alter the endogenous expression of N/OFQ and NOP receptor, notably in different areas of the hippocampus. Moreover, NOP antagonists have proven efficacious in attenuating anxiety-depressive symptoms in rats exposed to unpredictable chronic mild stress [47] and in patients suffering from major depressive disorder [28] but these studies did not assess cognitive symptoms. We suggest that a dysregulation of the N/OFQ system could be associated with chronic stress-induced memory deficits and that, in addition to anxiety-depressive symptoms, blocking NOP receptors could be beneficial for cognitive symptoms in stress-related disorders.

In order to probe this hypothesis, we have studied the impact of the pharmacological blockade of NOP receptors on long-term recognition and location memory deficits in a mouse model of chronic stress based on the prolonged administration of corticosterone. A second part of the study focused on the neuronal activity of the hippocampus and the role of the N/OFQ system in this brain region.

Materials and methods

Animals

Adult male C57BL/6J mice were purchased from Janvier Laboratories (Le Genest St. Isle, France) and allowed to acclimatize to the animal facility for at least one week before the beginning of the experiments. Heterozygous mice with floxed NOP receptor gene fused to Yellow fluorescent protein (YFP) on a C57BL6/J background (referred to as NOPR^{lox/lox}) were obtained from the University of Washington, Seattle, WA, USA (Prof Michael Bruchas; [18]). Homozygous NOPR^{lox/lox} mice were generated from the heterogeneous breeding pairs in our animal facility. The genotype was confirmed by PCR using a YFP and two NOP receptor gene (Opr11) primers. The WT allele resulted in a band of 176 bp and the mutant in a band of 241 bp. The animal protocol was approved by the local Ethics Committee and the French Ministry of Education and Research (APAFIS#27219-2020091610141233). All experiments conformed to the European guidelines for the care of laboratory animals (European Union Directive 2010/63/EU). Mice were housed at the animal facility with a 12h dark/light cycle (lights on at 8.00 am) in a temperature-controlled room (21°C) and had ad libitum access to water and food. All the behavioral experiments were carried out in the morning (from 8:00 am to 2:00 pm).

Stress induction procedures

Chronic corticosterone administration - Corticosterone (C2505, Sigma-Aldrich, St Quentin Fallavier, France) was diluted in 20% β -cyclodextrin (C4767, Sigma-Aldrich) and added to the drinking water for a final concentration of 35 mg/l of corticosterone (equivalent to 5 mg/kg/day) and 22,5 ml/l of β -cyclodextrin for 5 weeks before experiments. The corresponding vehicle consisted of 22,5 ml/ of 20% β -cyclodextrin.

Each cage was randomly allocated to a chronic treatment group (vehicle or CORT). Then within each cage, animals were randomly allocated to an acute treatment group (vehicle or NOP antagonist) or a virus treatment group (AAV5-hSyn-eGFP or AAV5-hSyn-GFP-Cre).

See Supplementary Methods for details on acute stress procedures.

Drugs

SB-612,111 (Axon, 1413, Groningen, The Netherlands) and compound 24 (C24; Axon, 1784) are two structurally unrelated highly selective NOP receptor antagonists with subnanomolar affinity [25]. They were administered i.p. at a dose of 10mg/kg. SB-612,111 was solubilized in 10% DMSO in a 0.9% NaCl solution, the corresponding vehicle (Veh) solution was 10% DMSO in 0.9% NaCl. C-24 was solubilized in 0.9% NaCl, the corresponding vehicle solution was 0.9% NaCl.

Injection of viral vectors

AAV5-hSyn-eGFP was obtained from Addgene (#50465, Cambridge, MA, USA). AAV5-hSyn-GFP-Cre and AAV5-hSyn-mCherry-Cre were obtained from the University of North Carolina Virus vector core. They had a titer from 2 to 5 10^{12} vg/ml and were kept at -80°C until use.

See Supplementary Methods for a description of the injection procedure.

For each mouse, GFP expression in the DG-CA3 region of both hemispheres was confirmed post-mortem on 30 μ m coronal section along the whole hippocampus.

Behavioral tests

See Supplementary Methods for details regarding behavioral assays, including Long-term location and recognition memory, Working memory and Short-term spatial memory, Open-field, Elevated plus-maze, Tail suspension, and splash tests.

Plasma corticosterone assay

See Supplementary Methods for details.

Immunostaining and in situ hybridization

See Supplementary Methods for details.

Images acquisition and analysis

See Supplementary Methods for details.

Data analysis

Graphics were generated and statistical analysis performed with GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA). Sample size were chosen based on previous experiments from our team. Data are presented as mean \pm SD. The results of all statistical analyses are presented in supplementary table 1. Normality was verified with the Shapiro-Wilk normality test and statistical tests were chosen accordingly. Comparisons to chance level were performed using one sample t test. Comparisons between two groups were performed using paired t-test, paired Wilcoxon, unpaired t-test or Mann-Whitney test depending on the normality of the data and the occurrence of repeated measures in the data set. Comparisons between more than 2 groups were performed using 1-way ANOVA followed by Tukey post hoc test or Kruskal-Wallis followed by Dunn post hoc test depending on the normality of the data.

Comparisons between two factors were performed using 2-way ANOVA or mixed-model Restricted Maximum Likelihood (REML) in case of repeated measures with missing values, followed by post-hoc Tukey analyses. Whenever a parametric test was used, a F test was performed to confirm the similarity of variances. Grubbs' method identified one outlier which was removed from the data presented in figure 3G (Veh_Veh group). The alpha risk was set at 0.05.

Results

NOP receptor antagonists impair long-term recognition and location memory in naïve mice

We first asked whether the blockade of the endogenous N/OFQ system would modulate memory performances in naïve unstressed animals. Mice were injected i.p. with a NOP antagonist, either SB-612,111 or compound 24 (C24), at the dose of 10 mg/kg and their performances were evaluated in several memory tests. For the object recognition and location tests, mice were injected with the compounds 15 minutes before the acquisition phase and their memory was tested 24h after acquisition (Fig. 1A). In the OR test acquisition phase, SB-612,111 but not C24 injection induced a decrease in the total exploration time (Suppl Fig. 1A). There was no preference for any object during the acquisition for any group (Suppl Fig. 1B). In the OL test, none of the antagonists affected the exploratory behavior (Suppl Fig. 1C) during the acquisition and there was no preference for any object during the acquisition for any group (Suppl Fig. 1D). In the OR test, only the vehicle group showed a significant preference for the novel object (Fig. 1B). Accordingly, the preference for the novel object was significantly reduced in the SB-612,111 and C24-injected mice compared to the vehicle-injected

group (Fig. 1C). Similarly, in the OL test, only vehicle-injected mice showed a significant preference for the displaced object (Fig. 1D). The preference for the displaced object was lower in both SB-612,111 and C24 groups compared to the vehicle group but only the difference between the vehicle and SB-612,111 reached statistical significance (Fig. 1E).

Impaired performances in the OR and OL tests could result indirectly from an alteration of the general behavior of the animals. We therefore performed additional behavioral tests to evaluate the impact of the SB-612,111 treatment on working and short-term spatial memory, and anxiety. In the Y-maze SA test, the SB-612,11 treatment had no impact on the total number of entries in the different arms meaning that the exploratory behavior was not modulated by the blockade of the NOP receptor (Fig. 1F). This blockade however slightly increased the performances of mice that did more correct alternations between the three arms than the vehicle-injected group (Fig. 1G). In the short-term spatial memory Y-maze test, the blockade of the N/OFQ-NOP system had no effect on performances, with both groups significantly preferring the previously closed-arm (Fig. 1H). The blockade of the NOP receptor had no effect on anxiety in the EPM test since mice spent a similar percentage of time in the open arms compared to vehicle-injected mice (Fig. 1I). Comparably, the vehicle- and SB-treated mice spent a similar amount of time in the center of the arena in the OF test (Fig. 1J). Overall these results suggest that acute treatment with NOP antagonists specifically impairs the establishment of long-term recognition and location memory.

NOP receptor antagonists restore long-term recognition and location memory in a chronic stress model

We then evaluated the ability of NOP receptor antagonists to modulate the deleterious effect of chronic stress on long-term recognition and location memory. Mice under chronic corticosterone treatment (CORT) were injected i.p. (10mg/kg) with SB-612,111 or C24 15 minutes before the acquisition session and their memory performances were assessed in the OR and OL tests. In all groups, there was no preference for any object in the OR (Suppl Fig. 2A) and in the OL acquisition phases (Suppl Fig. 2C). The corticosterone and NOP antagonist treatments did not modify the exploratory behavior during acquisition since there was no effect of treatment on the total exploration time in the OR (Suppl Fig. 2B) nor the OL (Suppl Fig. 2D) learning sessions. In the OR test, chronic CORT-exposed mice (CORT_Veh group) that did not receive any antagonist injection did not show a significant preference for the novel object (Fig. 2A). Furthermore, this group showed significantly less preference for the novel object than vehicle-treated control mice (Veh_Veh group) indicating a memory deficit (Fig. 2B). SB-612,111 or C24 administration restored the preference for the novel object (Fig. 2A) with a significant difference between the CORT_Veh group and mice exposed to CORT and treated with C24 (CORT_C24 group; Fig. 2B). Similar results were obtained in the OL test with the administration of C24 in mice chronically exposed to CORT. Both Veh_Veh and CORT_C24 groups showed a significant preference for the displaced object compared to the undisplaced one (Fig. 2C). Their preference for the displaced object was significantly higher than the one of the CORT_Veh group (Fig 2D).

We performed a Y-maze SA test with SB-612,111 to evaluate the potential impact of corticosterone and of the blockade of the N/OFQ-NOP system on spatial working memory. Neither the CORT nor the SB-612,111 treatment had an effect on the spontaneous alternation (Fig. 2E). Taken together, these results indicate that blocking

the endogenous N/OFQ system rescues long-term recognition and location memory deficits caused by chronic CORT treatment.

We then assessed whether the blockade of the NOP receptor could affect the memory consolidation process. We thus administered the NOP antagonist just after the acquisition phase of an OR memory test (Fig. 2F). During the acquisition phase, no group showed a preference for any object (Suppl Fig. 2E) and their exploratory behavior did not differ (Suppl Fig. 2F). As expected during the test session, CORT_Veh mice were the only one showing no preference for the novel object, indicating a memory deficit (Fig. 2G) and had a significantly lower percentage of preference for the novel object than Veh_Veh mice (Fig. 2H). Preference was higher in the CORT_SB than in the CORT_Veh group but this effect did not reach statistical significance ($p=0.07$). Thus, the NOP antagonist treatment favors long-term memory consolidation in the chronic stress model.

NOP receptor antagonists do not modulate anxiety and the response to acute stress in the chronic stress model

Memory deficits induced by CORT and/or the beneficial effect of NOP antagonists could be mediated indirectly by changes in the anxiety level of the animals or in the way they respond to novel stressful situations. We therefore studied anxiety and depressive-like symptoms, as well as the reactivity of the hypothalamic–pituitary–adrenal (HPA) axis to stressful stimuli, in our chronic stress model.

In the open-field performed during the OR and OL habituation session (i.e. before antagonist treatment), Veh_Veh and CORT_Veh mice spent the same percentage of time in the center zone (Fig. 3A) suggesting that the corticosterone treatment did not induce anxiety. We also studied the potential capacity of SB-612,111 to modulate

anxiety in CORT mice in the EPM test and found that SB-612,111 did not modulate the percentage of time in the open arms (Fig. 3B). To assess depressive-like symptoms, we performed a TST in which neither the corticosterone treatment nor the blockade of the N/OFQ-NOP system had an impact on the immobility time (Fig. 3C). Comparable results were obtained in the splash test in which grooming was not affected by CORT or the NOP antagonist (Fig. 3D). These results indicate that in our model, mice show no spontaneous anxiety and depressive-like activity and that SB-612,111 does not modulate significantly these behaviors.

We then investigated whether the NOP antagonist could modulate the HPA axis response in the chronic stress model. First, we used i.p. injection of vehicle or SB-612,111 as an acute stress and measured the plasma corticosterone concentration at different time points (Fig. 3E). As expected at T0, the basal level of corticosterone was higher in the two groups of mice exposed to chronic CORT compared with the vehicle-treated group (Fig. 3F). 15 minutes after stress, only the control group showed an increase in plasma CORT concentration followed by a return to basal level after 90 minutes. For both CORT-treated groups, plasma CORT concentration gradually decreased over the course of the experiment. As a result, there was a statistical difference in the relative levels of plasma CORT between the Veh_Veh group and the two CORT groups at the 15 and 90 minutes time points (Fig 3G). Therefore, in CORT mice, the HPA axis did not respond to the stressful stimulus and importantly, the SB-612,111 administration did not modify this hindered response to stress. To go further, we investigated a potential modulation of the neuronal response to stress by the N/OFQ system in our model. In particular, we studied neuronal activity using the immediate early gene c-Fos in the paraventricular nucleus of the hypothalamus (PVN). Mice received an i.p. injection of SB-612,111 or vehicle 30 minutes before a 6 minutes

session of forced swimming and were sacrificed 90 minutes later. Both CORT groups showed a significant decrease in the number of c-Fos+ cells compared to the Veh_Veh group indicating a lower PVN activity following acute stress. More importantly, there was no difference between the two CORT groups suggesting that the blockade of the N/OFQ system did not modulate the neuronal response to stress in the PVN (Fig. 3H, I). Overall, we observed a blunting of the HPA axis response to stress in CORT mice that was not affected by the NOP antagonist.

Altogether, these results indicate that memory deficits induced by chronic CORT and the beneficial effects of NOP antagonists cannot be attributed to an indirect modulation of anxiety-depression symptoms or of the HPA axis-mediated response to stress.

Endogenous N/OFQ expression is increased in the hippocampus following chronic CORT exposure.

Since behavioral data were pointing towards a direct modulation by NOP antagonists of long-term memory, and in particular of memory consolidation, we focused the second part of the study on the hippocampus.

We first performed in-situ hybridization to evaluate the effect of chronic CORT exposure on N/OFQ precursor and NOP receptor expression. Suppl Fig 3 shows the expression pattern of the two mRNAs in the hippocampus. Consistent with the literature, ppN/OFQ expression was restricted to interneurons whereas NOP receptor mRNA was found in the principal cells of the dentate gyrus (DG), CA3, CA2, and CA1 subregions as well as in a lower number of interneurons. We assessed the level of ppN/OFQ and NOP mRNAs expression in mice treated either with vehicle or CORT for six weeks. The density of cells expressing the N/OFQ precursor mRNA was significantly higher in the CORT-treated group compared with the vehicle-treated group

in every subregion of the hippocampus analyzed (Fig. 4A-D). The average intensity of the NOP mRNA signal was higher in every subregion of the hippocampus analyzed but this increase did not reach statistical significance (Fig. 4E-H). Therefore, it appears that chronic CORT mainly increases the expression of the N/OFQ-NOP system in the hippocampus at the level of the neuropeptide precursor.

Chronic CORT and NOP antagonist treatment have opposite effects on neuronal activity in the CA3 region of the hippocampus

We then investigated the potential modulatory effect of the endogenous N/OFQ-NOP system on hippocampus activity (c-Fos expression), under our behavioral experimental conditions.

Half of the Veh and CORT animals were treated with 10 mg/kg of SB-612,111 15 minutes before being exposed to a 10 minutes OR-like acquisition session and sacrificed 90 minutes after. Compared to vehicle-treated mice, the density of c-Fos positive cells was significantly increased in the principal cell layers of the CA3 subregion (Fig. 5A, C) and the DG (Fig. 5B) of the hippocampus of chronic CORT mice, but not in the CA1 region (Fig. 5D). In the DG and CA1 subregions, there was no effect of the blockade of the N/OFQ-NOP system (Fig 5B, D). Interestingly, in the CA3 subregion, the SB-612,111 treatment resulted in a decrease in the density of c-Fos-positive cells regardless of the chronic treatment conditions (Fig 5A, C).

Altogether, these results indicate that in the chronic stress model, the size of the neuronal ensembles that are activated in the DG and CA3 subregion of the hippocampus following exposure to new objects increases. Moreover, blocking the NOP receptor decreases neuronal activity in CA3.

Conditional knockout of NOP receptor in the hippocampus is sufficient to prevent chronic CORT induced long-term recognition and location memory deficits

In order to confirm that the promnesic effects of the NOP antagonists were indeed due to the blocking of the receptor in the hippocampus, we knocked out the NOP receptor in this region. In light of the above c-Fos data, we specifically targeted the DG/CA3 region. NOPR^{lox/lox} mice were injected with either an AAV5-hSyn-eGFP-Cre or an AAV5-hSyn-eGFP viral vector as a control. They were then exposed to CORT or vehicle for 5 weeks before being tested for long-term OR and OL memory (Fig. 6A). We used in-situ hybridization to confirm that the AAV5-hSyn-eGFP-Cre injection induced a strong decrease in NOP receptor mRNA expression in the DG/CA3 region (Fig. 6B). Since the NOP receptor is fused to YFP in NOPR^{lox/lox} mice, we injected an AAV5-hSyn-mCherry-Cre virus in another group of animals to confirm that our strategy resulted in a strong diminution of the NOP-YFP protein signal in the DG/CA3 region (Suppl Fig. 4).

The CORT groups showed a decrease in the total exploration time during the OR acquisition phase (Suppl Fig. 5A). No group showed a preference for any object during acquisition (Suppl Fig. 5B). In the OR test, apart from the CORT-treated mice that had received the control virus injection, every group spent significantly more time exploring the novel object compared with the familiar one (Fig. 6C). Accordingly, NOP receptor KO did not affect the preference for the novel object in the vehicle-treated mice but restored it in the CORT-treated animals (Fig. 6D). Altogether these results indicate that NOP receptor KO in the DG/CA3 region has no impact on recognition memory in vehicle-treated mice but restores memory performances in CORT-treated animals.

During the OL acquisition phase, neither CORT treatment nor receptor KO had an impact on the exploration time (Suppl Fig. 5C) and no group showed a preference for any object (Suppl Fig. 5D). In the OL test, mice chronically exposed to vehicle after control virus injection and mice chronically exposed to CORT after AAV5-hSyn-eGFP-Cre injection showed a significant preference for the displaced object (Fig. 6E). Accordingly, NOP receptor KO significantly reduced the preference for the novel object in the vehicle-treated mice but restored it in the CORT-treated animals (Fig. 6F). Altogether, these results indicate that NOP receptor KO in the DG/CA3 region has a deleterious impact on object location memory in vehicle-treated mice but restores memory performances in CORT-treated mice in the object location test.

Discussion

Here we report opposite effects of the pharmacological blockade of the NOP receptor on long-term object recognition and location memory in naïve mice and in mice exposed to corticosterone. Two structurally unrelated antagonists were used to confirm that those effects were specific to the NOP receptor. They impaired long-term object recognition and location memory in naïve mice, whereas they rescued memory deficits in the chronic stress model. Therefore, it appears that endogenous N/OFQ release during the process of episodic-like memory encoding directly modulates memory performances. In control mice, the activity of the endogenous N/OFQ system is necessary for OR and OL memory whereas in the neuroendocrine chronic stress model it is deleterious.

In the present study, we adjusted the duration of the CORT treatment in order to reveal cognitive deficits without interference from anxiety/depression symptoms associated

with this model [3, 48]. The HPA axis reactivity is altered in our model but, importantly, this alteration is not affected by SB-612,111 treatment. Therefore, the modulation of memory performances by the compounds cannot be attributed to an indirect modulation of anxiety-depression symptoms or of the stress response.

Although a large number of studies have described amnesic effects of N/OFQ [34, 49], suggesting a promnesic impact of NOP antagonists, here we demonstrate unexpectedly a deleterious effect of NOP antagonists on episodic-like memory in naive mice. However, two studies have reported biphasic effects of exogenous N/OFQ administration on memory in healthy rodents. Intra-hippocampus infusion of a high dose of N/OFQ in rats impairs memory in the Morris Water Maze whereas lower doses facilitate it [50]. Similarly in mice, i.c.v. injection of a low dose of N/OFQ improves inhibitory avoidance memory whereas high doses have a negative effect on memory performance [51]. Considering this putative inverted-U-shaped relationship between N/OFQ activity and memory performance, we hypothesize that chronic corticosterone induced an upregulation of the N/OFQ system detrimental for memory. This is consistent with the increased expression of N/OFQ in the hippocampus observed in the present work, but also in a previous study that used a chronic social crowding model [45]. Beside memory modulation, endogenous N/OFQ is believed to contribute to the adaptive response to acute stress, notably because of its anxiolytic properties [29, 52]. Our data suggest that it could also be important for the adaptation to novel situations, by improving the encoding of relevant memories. On the contrary, the N/OFQ system has been shown to play a role in maladaptation to traumatic or chronic stressors, promoting pain, anxiety and depressive symptoms [27, 47, 53–55]. We show here that endogenous N/OFQ could also contribute to cognitive deficits triggered by chronic stressors [56–58]. Overall, our data demonstrate that endogenous N/OFQ

modulates cognitive processes in opposite ways depending on the history of stress hormone exposure.

Since object recognition and location memories are highly dependent on the hippocampus [59], a major target of glucocorticoids [4, 60], we focused on this region to better understand the behavioral consequences of N/OFQ-NOP system endogenous signaling. We thus tested whether the conditional knock-out of the NOP receptor in the DG-CA3 region could recapitulate the effect of systemic antagonist administration. This was only partially the case in naïve mice in which NOP KO induced memory deficits in the OL but not in the OR test. NOP receptor signaling in the hippocampus circuit is therefore necessary for object location memory whereas it appears dispensable for object recognition. This observation echoes the debate on the role of the hippocampus in recognition memory [59, 61, 62]. We hypothesize that the discernment of familiarity which depends on the perirhinal cortex, was sufficient for the mice to succeed in the OR task despite the disruption of hippocampal function induced by the NOP receptor KO. In the chronic stress model, hippocampal NOP receptor KO mimicked the effect of systemic NOP antagonists in both the OR and OL test. This suggests that chronic exposure to corticosterone leads to changes in hippocampal circuitry making the local N/OFQ system no longer permissive but, on the contrary, deleterious for the establishment of episodic-like memories.

Thus, endogenous N/OFQ appears to impair the memory consolidation process after chronic stress. Despite those encouraging results, the mechanism(s) underlying this memory impairment remain elusive. CA3 circuits are thought to play a key role in the storage of associative memories [63]. One trial associative learning relies on activity-dependent plasticity of ensembles of CA3 pyramidal cells driven by sparse inputs from DG granule cells [63]. This phenomenon is modulated by the activity of several

subtypes of interneurons that control memory engrams, in particular the size of neuronal memory ensembles [63–65]. Our in situ hybridization analyses was consistent with ppN/OFQ being expressed in hippocampal interneurons [32, 66] whereas NOP receptor mRNA was found in the principal cells of all subregions as well as in some interneurons [12, 31]. N/OFQ could therefore contribute to the control of the activity-dependent plasticity of CA3 principal cells and shape the size of neuronal ensembles underlying long-term memory. This is consistent with our c-Fos analyses which revealed a decrease in the density of activated neurons following exposure to the objects in mice treated with the NOP antagonist. In naïve mice, this decrease in neuronal activation in CA3 could underly memory deficits in the OL task. In the hippocampus, chronic stress has been shown to result in a decline in neuronal ensemble coherence [67], and an alteration in the firing of principal cells and in neural oscillations [68]. Here, we observed an increase in the size of neuronal ensembles associated with memory encoding in mice exposed to chronic corticosterone. Interestingly, NOP antagonist treatment hindered this increase in the CA3 region, normalizing the density of c-Fos+ cells to the level observed in control mice. Cognitive symptoms associated with chronic stress have been proposed to result from an altered signaling of GABAergic interneurons resulting in defects in the inhibition/excitation balance in cortical and subcortical microcircuits [69, 70]. Our data suggest that N/OFQ released from hippocampal interneurons contributes to these alterations. In CORT-exposed mice, blocking NOP receptors normalizes the density of active cells in the CA3 region. Therefore, NOP antagonist treatment could restore an optimal inhibition/excitation balance for memory encoding and consolidation. In vivo and in vitro studies have suggested that N/OFQ acted on memory processes through the regulation of glutamate signaling in the hippocampus [34, 38, 41] but N/OFQ also

affects acetylcholine release in this region [71, 72]. On brain slices, N/OFQ decreases activity and plasticity of principal hippocampal neurons [34]. Thus, one would anticipate that silencing NOP receptor signaling would increase hippocampal activity and plasticity. While our data demonstrate that NOP receptor antagonist decreases hippocampal activity, further studies are warranted to describe in more detail the distribution of N/OFQ and its receptor in the different subtypes of hippocampal neurons before proposing a comprehensive model of the regulation of hippocampal microcircuits by the neuropeptide.

We acknowledge a number of limitations in our study. First, it was performed in a pharmacological model of chronic stress. The chronic corticosterone model shows strong construct and face validity in terms of hippocampal structural and functional alterations as well as of cognitive deficits [3, 48, 73, 74]. Moreover, it has been shown that acute corticosterone administration could mimic the effect of restraint stress on hippocampal N/OFQ expression [43]. However, it would be important to confirm our results in another mouse model of chronic stress. Second, our study was performed only on male mice. Sex differences in behavioral response to chronic oral corticosterone, as well as in hippocampal epigenetic signatures, have been described recently [75]. Moreover, the N/OFQ system has been shown to adapt differently in male and female rats following traumatic stress [76]. It would therefore be important to include female mice in a follow-up study.

In conclusion, our findings reveal opposite roles of the endogenous N/OFQ system on long-term object recognition and location memory in naïve mice (for which it is necessary) and in mice exposed to chronic corticosterone (for which it is deleterious). We also demonstrate that this modulatory role is mediated at least in part by the NOP receptor expressed in hippocampal microcircuits. We suggest that NOP antagonists

which are currently under clinical development [77] could represent a viable option for the treatment of cognitive symptoms associated with stress-related disorders. However, their efficacy might depend on the magnitude of the stress-induced changes in the N/OFQ system in individual patients over the course of their entire life, prompting to the characterization of the in vivo status of N/OFQ and NOP receptors in individuals that have been exposed to chronic or traumatic stress.

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Figure legends

Figure 1: Effect of NOP receptor antagonists on memory. A, Schematic drawing of the OR and OL protocols: antagonist (10 mg/kg) or vehicle solution is injected i.p. 15 min before the learning phase and the memory is tested the next day. B, Contrary to the Vehicle group, SB-612,111- and C24-injected mice did not spend more time exploring the novel object compared to the familiar one during the OR test (n= 10-23 mice/group; ***p<0.001; paired t-test). C, SB-612,111- and C24-injected mice showed less percentage of preference for the novel object than Vehicle-injected mice in the OR test (n= 10-23 mice/group; **p<0.01 and *p<0.05; Kruskal-Wallis followed by Dunn's multiple comparisons test). D, Contrary to the Vehicle group, SB-612,111- and C24-injected mice did not spend more time exploring the displaced object compared to the undisplaced one during the OL test (n= 7-21 mice/group; ***p<0.001; paired t-test). E, There was a significant difference between Vehicle and SB-612,111 groups in the percentage of preference for the displaced object during the OL test (n= 7-21 mice/group; *p<0.05; One-way ANOVA followed by Tukey's multiple comparisons test). F, The SB-612,111 injection had no impact on the number of entries in the arms during the spontaneous alternation test (n=10 mice/groups; unpaired t-test). G, The antagonist injections induced an increase in the percentage of alternation between the arms in the spontaneous alternation test (n=10 mice/group; *p<0.05; unpaired t-test). H, The blockade of the N/OFQ-NOP system had no impact on the percentage of entries in the new arm during the closed-arm test (n=10 mice/group; ###p<0.001; one-sample t-test compared to 33%). I, Blocking the N/OFQ-NOP system did not modify the behavior of mice in the EPM (n=10 mice/group; unpaired t-test). J, Blocking the N/OFQ-NOP system did not modify the behavior of mice in the OF (n=10 mice/group; unpaired t-test). Data are expressed as mean \pm SD.

Figure 2: Effect of NOP receptor antagonists on long-term recognition and location memory deficits in the chronic stress model. A, Apart from the CORT_Veh group, all groups spent more time exploring the novel object compared to the familiar one during the OR test (n= 9-24 mice/group; ***p<0.001 and **p<0.01, paired Wilcoxon or t-test). B, SB-612,111- and C24-treated mice presented higher percentage of preference for the novel object than Veh-injected CORT mice in the OR test (n= 9-24 mice/group; ***p<0.001; Kruskal-Wallis followed by Dunn's multiple comparisons test). C, Apart from the CORT_Veh group, all groups spent more time exploring the displaced object compared to the undisplaced one during the OL test (n= 14-15 mice/group; ***p<0.001, paired Wilcoxon and t-test). D, C24-treated mice presented a higher percentage of preference for the novel object than Vehicle-injected CORT mice in the OL test (n= 14-15 mice/group; *p<0.05 and ***p<0.001; Kruskal-Wallis followed by Dunn's multiple comparisons test). E, Neither treatment modulated mice working memory performances in the Y-maze (n=10 mice/group, Kruskal-Wallis). F, Schematic drawing of the OR protocol: antagonist (10 mg/kg) or vehicle solution was injected i.p. right after the acquisition phase and the memory was tested the next day. G, Apart from the CORT_Veh group, all groups spent more time exploring the novel object compared to the familiar one during the OR test (n= 14-15 mice/group; ***p<0.001 and **p<0.01, paired Wilcoxon and t-test). H, SB-612,111 mice presented a higher percentage of preference for the novel object than Veh-injected CORT mice in the OR test (n= 14-15 mice/group; **p<0.01; Kruskal-Wallis followed by Dunn's multiple comparisons test). Data are expressed as mean \pm SD.

Figure 3: Lack of modulation of anxiety, depressive-like behavior, and HPA axis reactivity by the NOP antagonist in the chronic stress model. A, The chronic CORT treatment did not modulate the anxiety of mice in the open field (n=30-59 mice/group; Mann-Whitney). B, Neither CORT treatment nor N/OFQ-NOP system blockade modulated the anxiety of mice in the elevated plus maze (n=8-10 mice/group; One-Way ANOVA). C, Neither CORT treatment nor N/OFQ-NOP system blockade had an impact on mice immobility in the TST (n=9-10 mice/group; One-way ANOVA). D, Neither CORT treatment nor N/OFQ-NOP system blockade had an impact on the grooming behavior in the splash test (n=8-10 mice/group; One-way ANOVA). E, Schematic drawing of the plasma corticosterone assay procedure. Blood samples were taken at T0 and then 15 and 90 minutes after i.p. injection. F, The plasmatic CORT level was higher in both CORT groups at T0 (n=7-8 mice/group; **p<0.01; Kruskal-Wallis followed by Dunn's test). G, The plasmatic CORT level of CORT groups was not increased after acute stress (n=6-8 mice/group; *p<0.05, Veh_Veh vs CORT_Veh and CORT_SB; REML followed by Tukey's test). H, Representative images at x20 magnification of c-Fos+ cells in the PVN of mice from each treatment group; scale bar 20µm. I, The density of c-Fos+ cells in the PVN following acute stress was lower in both CORT groups compared to Vehicle-treated mice (n=6 mice/group; *p<0.05; One-way ANOVA followed by Tukey's test). Data are expressed as mean ±SD.

Figure 4: Modulation of N/OFQ precursor and NOP receptor mRNA expression by chronic CORT. A, Representative images of N/OFQ precursor mRNA signal (green) in the DG, CA3, and CA1 (from left to right) after vehicle (top) and CORT (bottom) treatment. Scale bar 30 µm. B, The density of cells expressing N/OFQ precursor

mRNAs is increased in the DG of CORT-treated mice (n=7-8 mice/group; **p<0.01; unpaired t-test). C, The density of cells expressing N/OFQ precursor mRNAs is increased in the CA3 of CORT-treated mice (n=7-8 mice/group; ***p<0.001; unpaired t-test). D, The density of cells expressing N/OFQ precursor mRNAs is increased in the CA1 of CORT-treated mice (n=7-8 mice/group; *p<0.05; unpaired t-test). E, Representative images of NOP receptor mRNA signal (red) in the DG, CA3 and CA1 (from left to right) of control mice. Scale bar 40 μ m. F, The average intensity of the NOP receptor mRNA signal is not different between the two groups in the DG (n=7-8 mice/group; unpaired t-test). G, The average intensity of the NOP receptor mRNA signal is not different between the two groups in the CA3 (n=7-8 mice/group; unpaired t-test). H, The average intensity of the NOP receptor mRNA signal is not different between the two groups in the CA1 (n=7-8 mice/group; unpaired t-test). Data are expressed as mean \pm SD.

Figure 5: Modulation of neuronal activity in the hippocampus following memory acquisition after chronic CORT and acute SB-612,111 treatment. A, Representative images of c-Fos labelled cells in the CA3 subregion for the Veh_Veh (top, left), the Veh_SB (top, right), the CORT_Veh (bottom, left), and the CORT_SB (bottom, right) groups. Dotted lines delimit the principal cell layer. Scale bar 20 μ m. B, CORT treatment induced an increased density of c-Fos positive cells in the DG (n=6-7 mice/group; *p<0.05; Two-way ANOVA). C, In the CA3 region, CORT treatment induced an increased density, and NOP antagonist administration induced a decreased density of c-Fos positive cells (n=6-7 mice/group; *p<0.05, CORT effect; #p<0.05, SB effect; Two-way ANOVA). D, Neither CORT treatment nor NOP receptor blockade had an

effect on the density of c-Fos positive cells in the CA1 subregion (n=6-7 mice/group; Two-way ANOVA). Data are expressed as mean \pm SD.

Figure 6: Conditional knockout of NOP receptor in the hippocampus restores long-term recognition and location memory performances following chronic CORT exposure. A, Schematic drawing of the experimental procedure. B, Representative images of the NOP mRNAs (red) in the CA3 subregion of the hippocampus of a non-injected (sham) mouse (left image) and a mouse injected with the AAV5-hSyn-eGFP-Cre virus (right image) where Cre-expressing cells are green. C, Apart from the CORT_no Cre group, all groups spent more time exploring the novel object compared to the familiar one during the OR test (n=9-13 mice/group; ***p<0.001, paired Wilcoxon or t-test). D, The local knock-out had no effect on the percentage of preference of vehicle-treated mice but restored a preference for the novel object in CORT-treated mice (n=9-13 mice/group; *p<0.05; Two-way ANOVA followed by Sidak's multiple comparisons test). E, Mice from the no CORT_no Cre and CORT-Cre groups spent significantly more time exploring the displaced object compared with the undisplaced one (n=11-12 mice/group; *p<0.05, **p<0.01, paired Wilcoxon or t-test). F, Cre decreased preference for the displaced object in the no CORT group but increased it in the CORT group during the OL test (n=11-12 mice/group; *p<0.05, **p<0.01, ***p<0.001; Two-way ANOVA followed by Tukey's multiple comparisons test). Data are expressed as mean \pm SD.

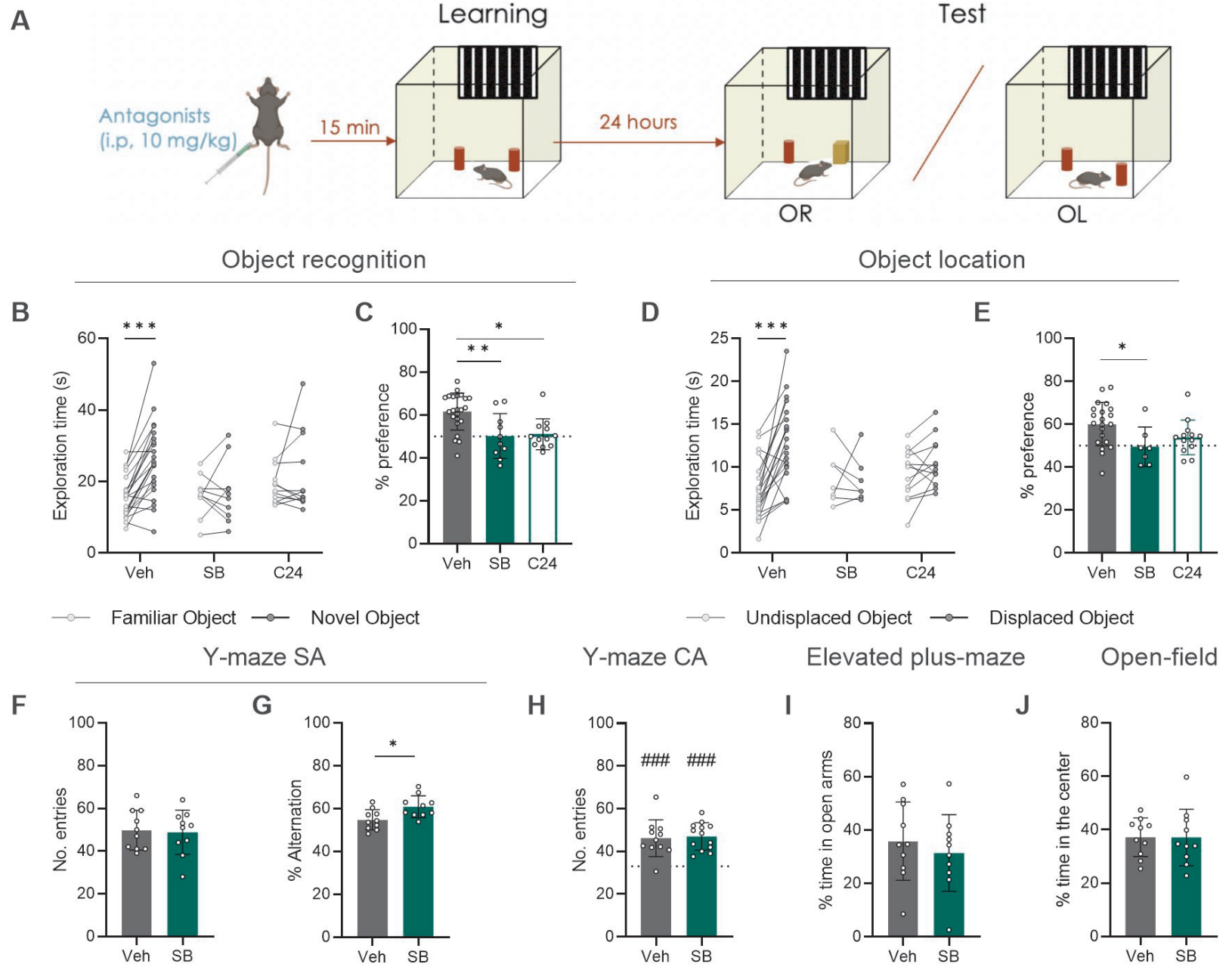
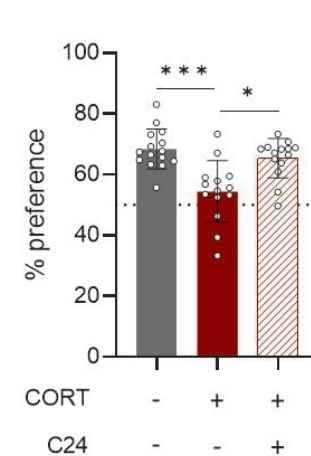
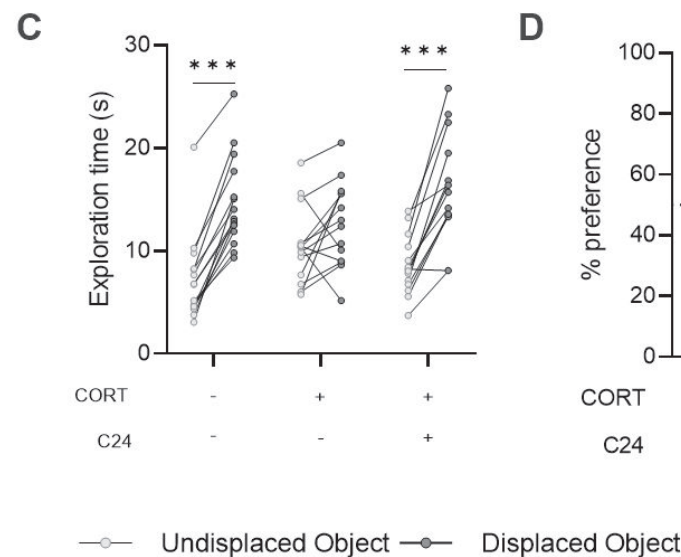
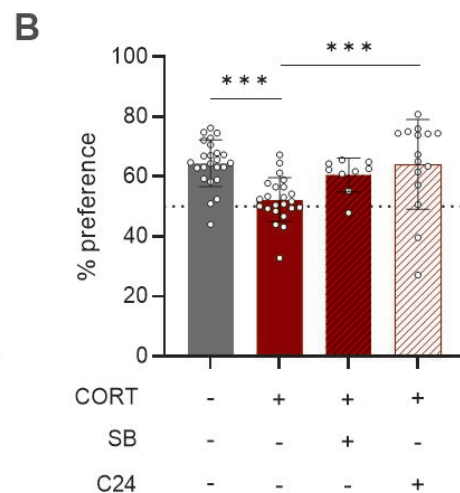
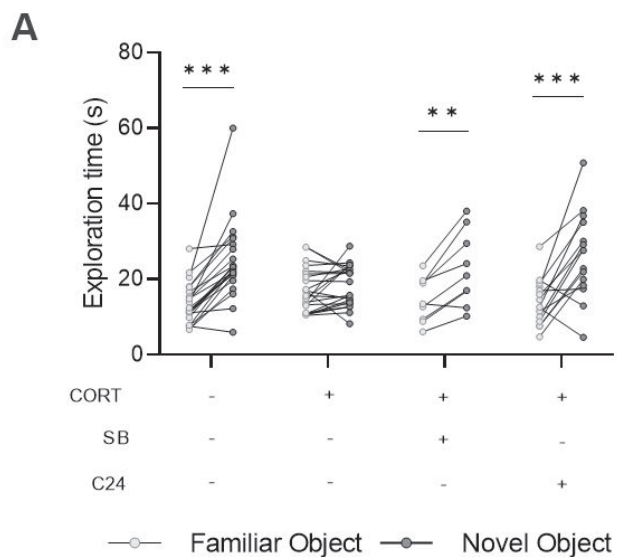


Figure 1

Object recognition

Object location



Y-maze SA

Object recognition

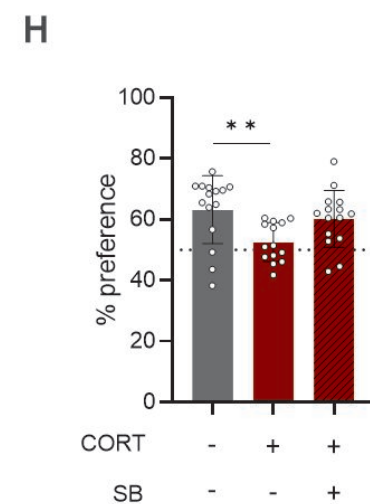
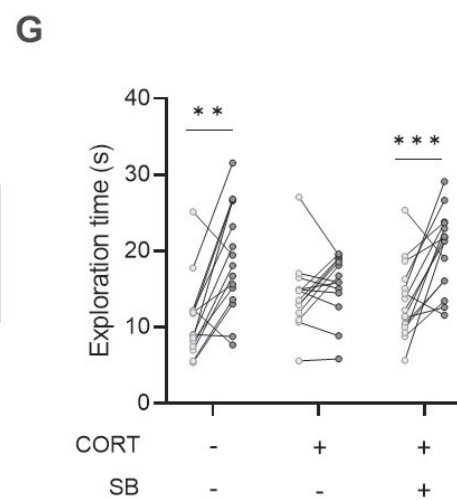
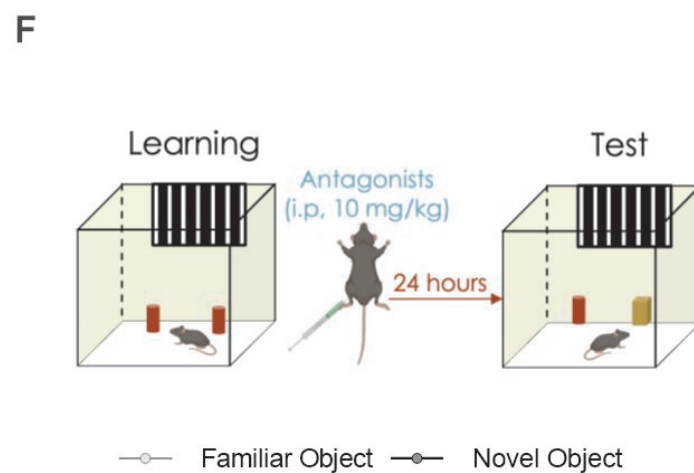
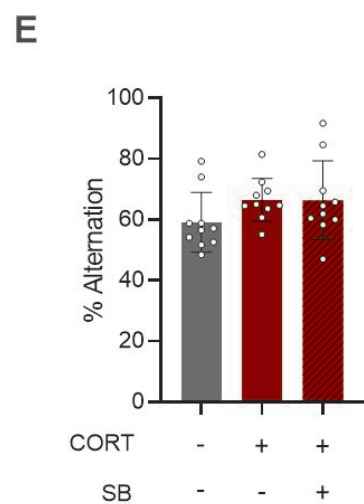


Figure 2

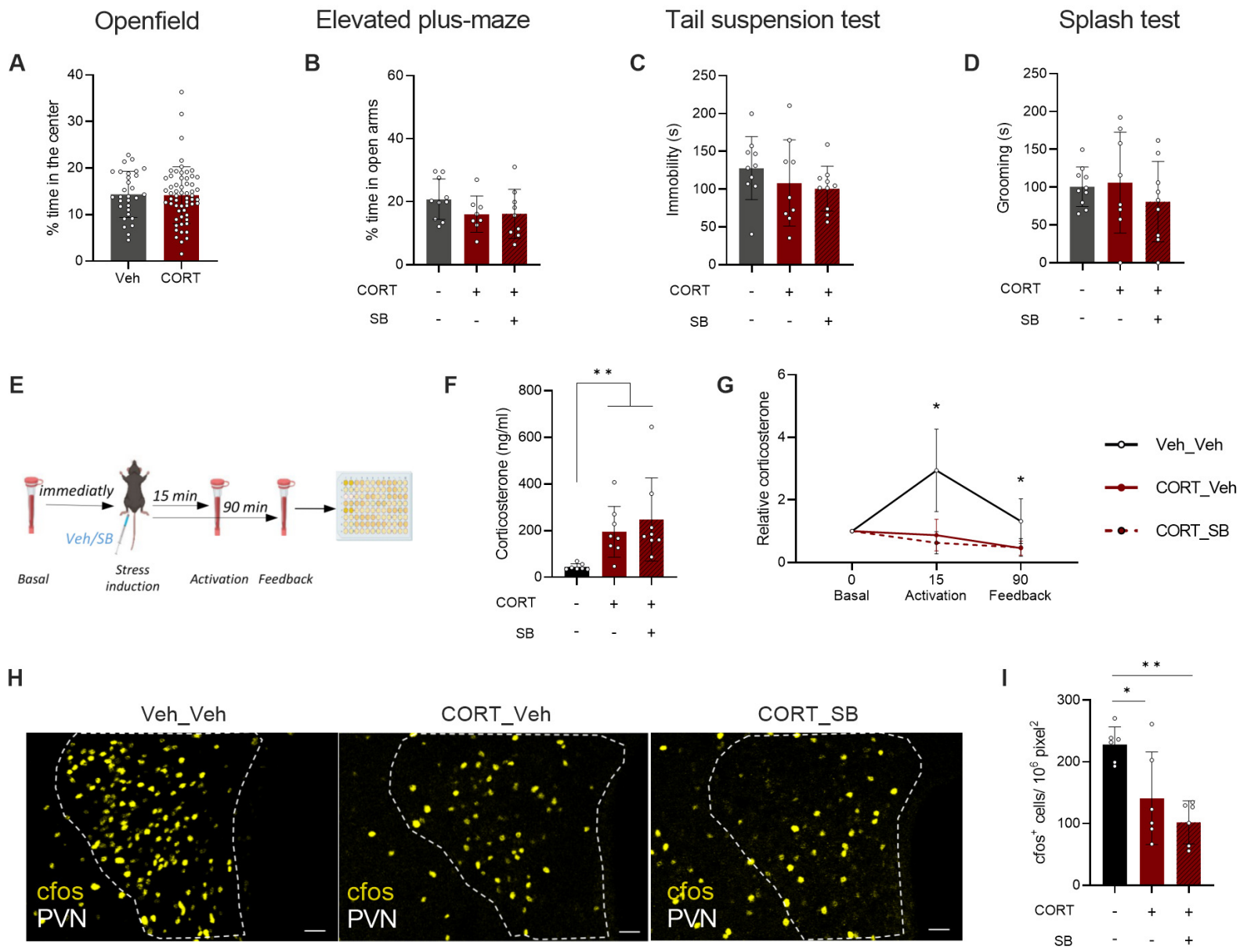


Figure 3

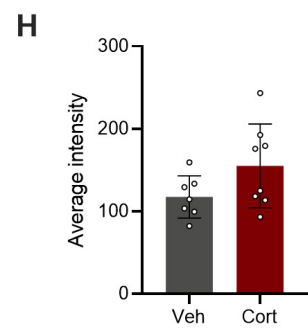
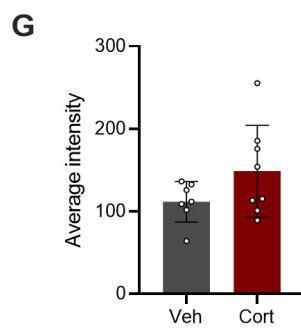
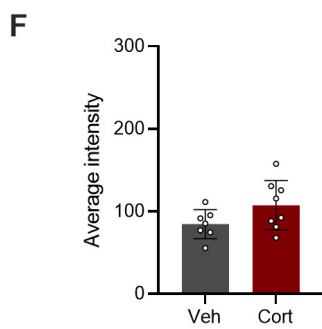
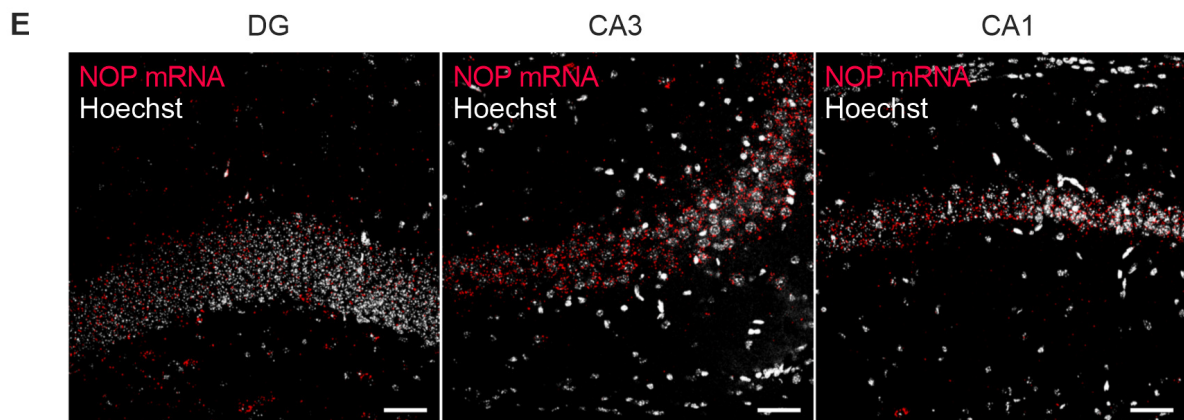
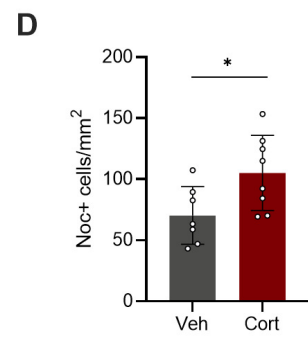
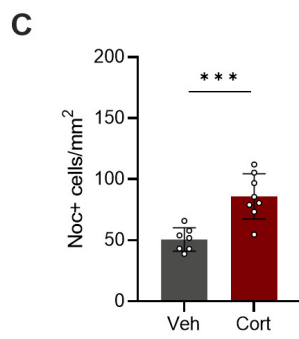
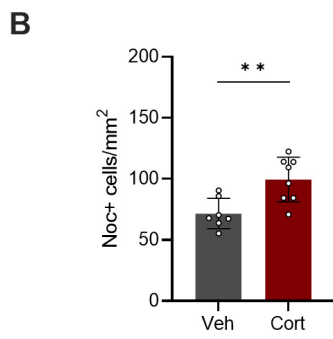
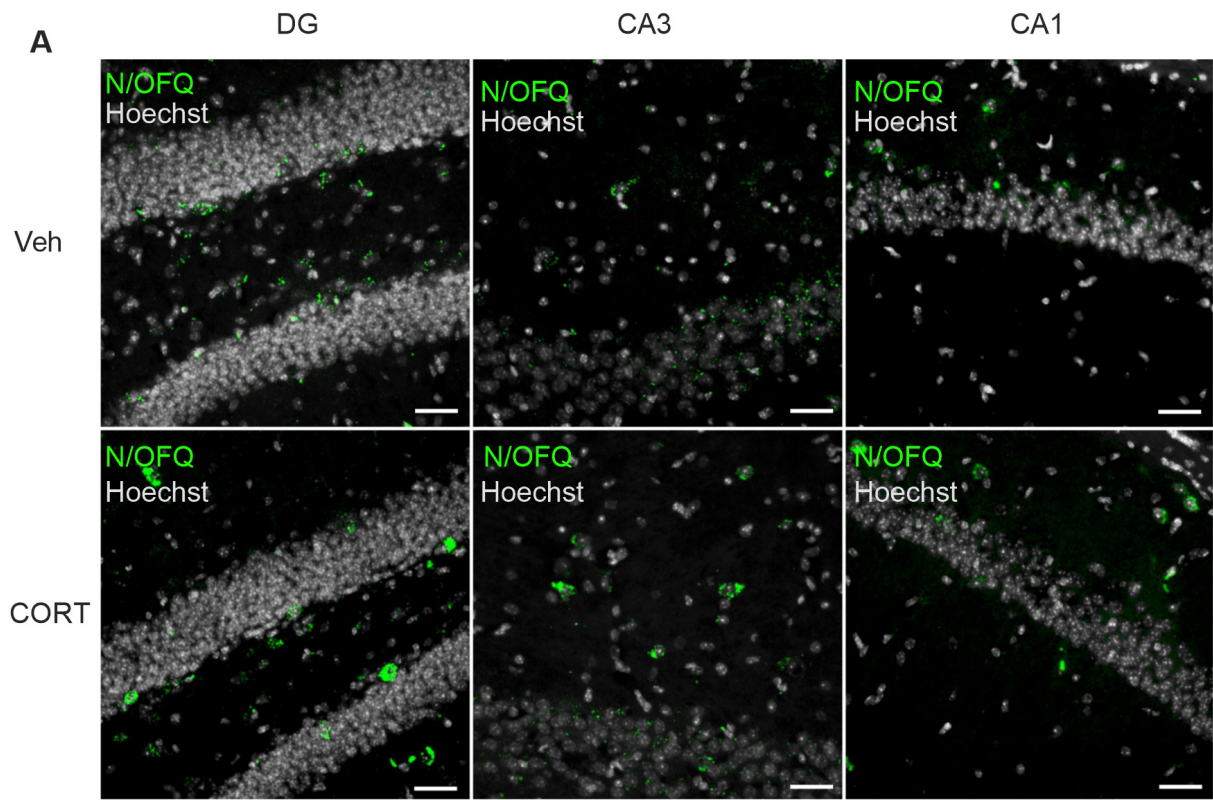


Figure 4

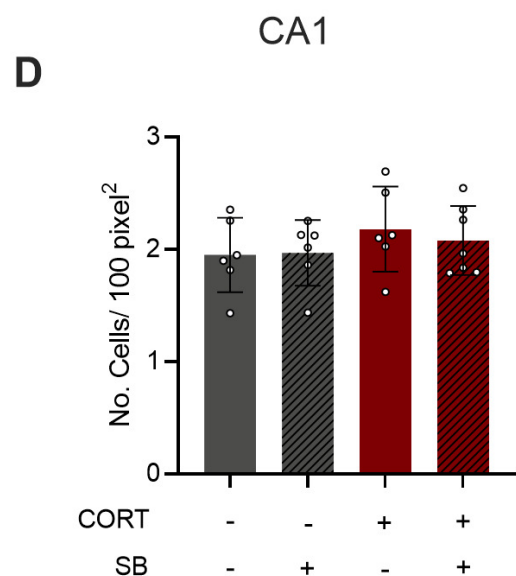
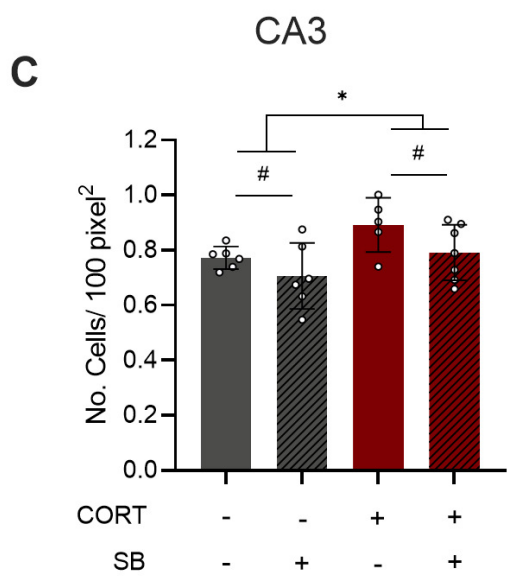
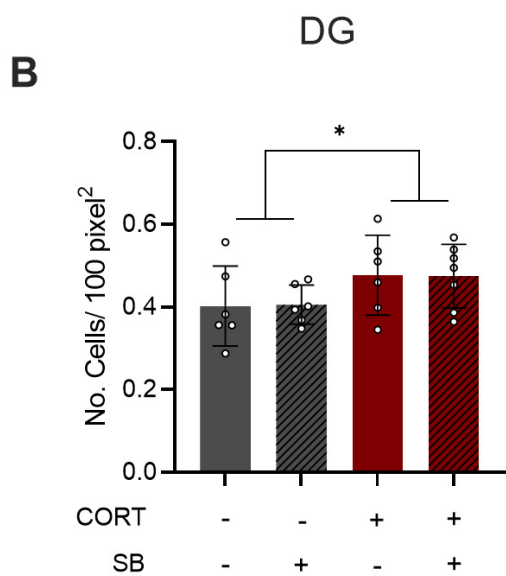
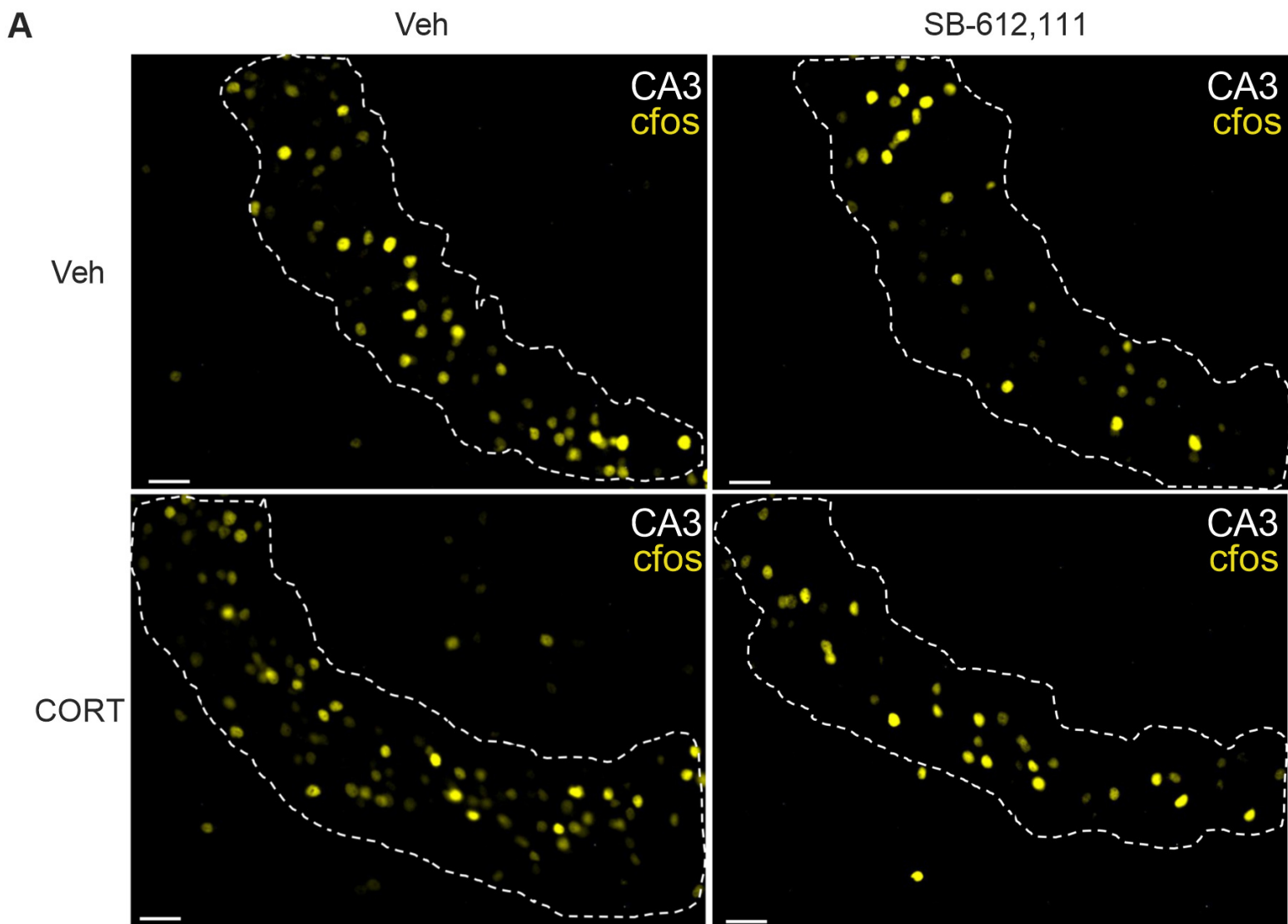
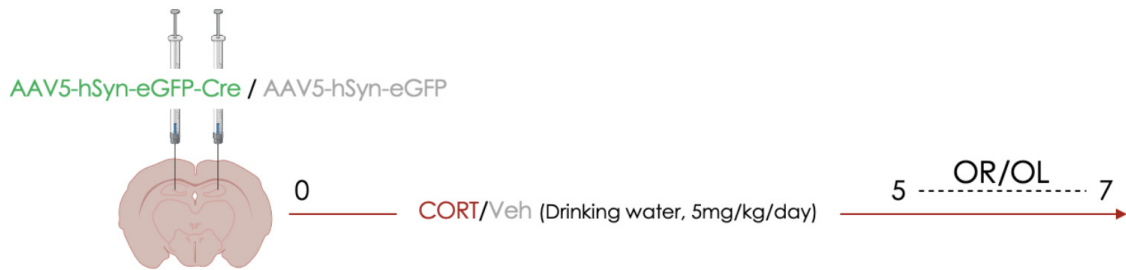
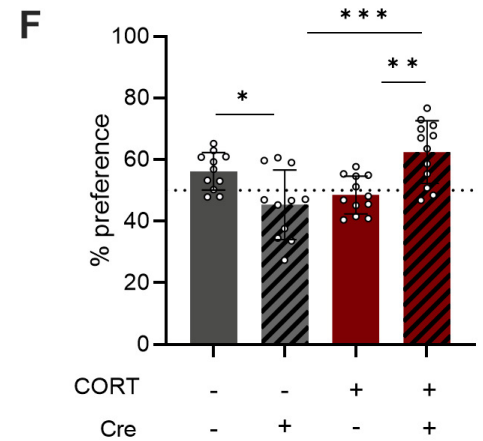
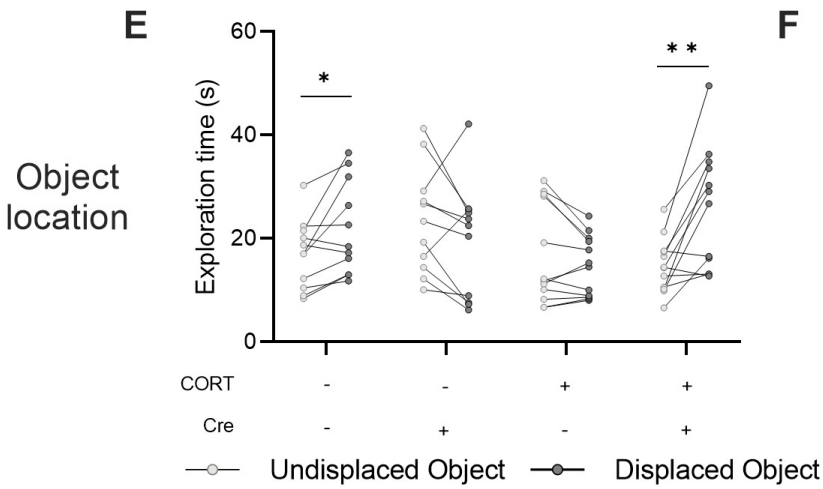
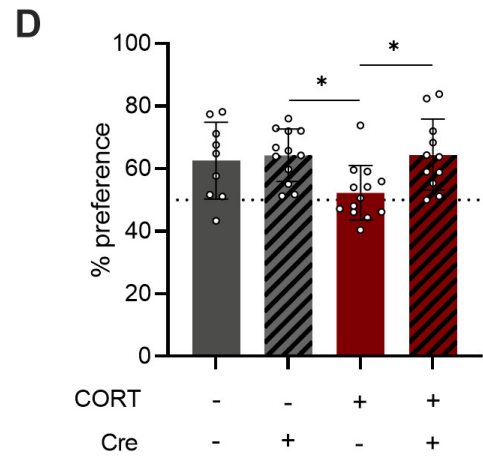
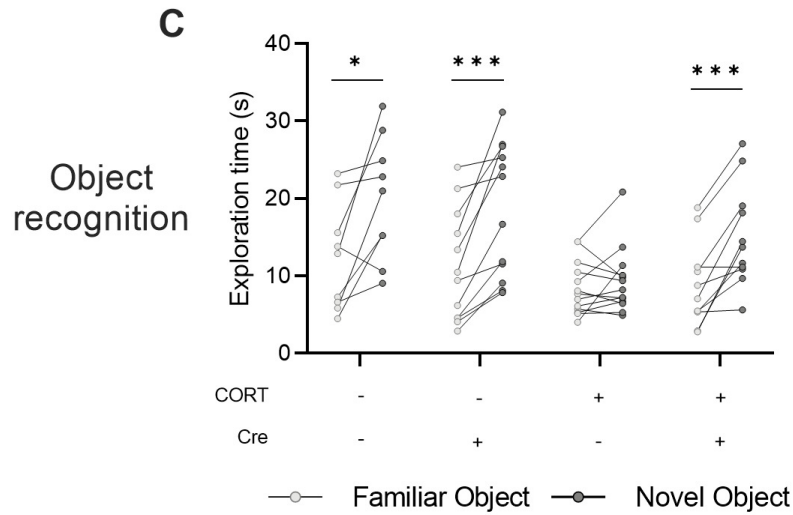
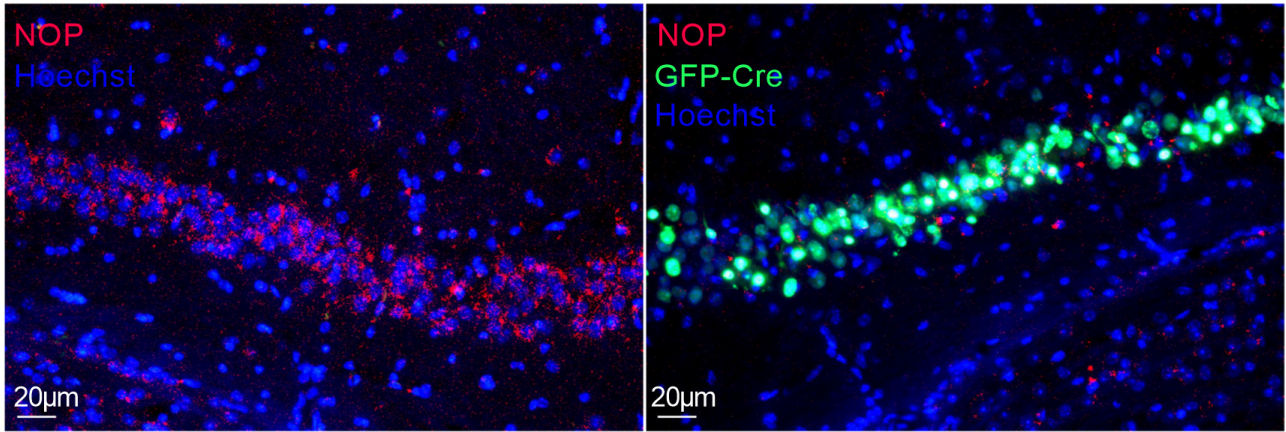


Figure 5

A**B****Figure 6**