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Social experience is associated with a differential role of aromatase neurons in sexual behavior and territorial aggression in male mice

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ABSTRACT

Aromatase (Aro+) neurons located in the Bed Nucleus of the Stria Terminalis (BNST) are crucial for the display of both sexual behavior and territorial aggression in naive male mice. The postero-dorsal part of the Medial Amygdala (MeApd) also contains Aro + neurons that are required for territorial aggression, but these neurons seem dispensable for the display of sexual behavior in naive animals. However, little is known about how Aro + neuron circuitry is influenced by social experience. Using a combination of chemogenetics, activity mapping and retrograde viral tracing, we show that social experience modulates Aro + neurons during sexual behavior and territorial aggression. Chemogenetic inhibition of BNST Aro + neurons in socially experienced male mice revealed that these neurons are required for territorial aggression, but not for sexual behavior. Behavior testing in experienced animals showed a specific increase in activation in the vomeronasal organ (VNO) and the Medial Amygdala (MeA) after sexual behavior but not territorial aggression, assessed by Egr1 expression. We also observed an increase of Egr1 cells in the medial Preoptic Area (mPOA), a brain region implicated in the display of sexual behavior. Combined retrograde viral tracing and Egr1 immunodetection showed that a subset of the activated cells in the MeA are Aro + neurons projecting to the mPOA. These results highlight that social experience induces a differential neural activity in the circuitry controlling sexual behavior and aggression, which include MeA Aro + neurons projecting to the mPOA.

1. Introduction

Sexual behavior and territorial aggression are two innate social behaviors that are controlled by hard-wired circuits linking olfactory inputs to stereotyped motor outputs (Lischinsky and Lin, 2020; Wei et al., 2021). The display of sexual behavior and territorial aggression are controlled by the social behavior network (SBN), a set of interconnected hormone-responsive brain areas that regulate several social behaviors. The SBN includes the principal nucleus of the Bed Nucleus of the Stria Terminalis (BNSTpr), the medial preoptic area (mPOA), the Medial Amygdala (MeA), the Ventromedial Hypothalamus (VMH) and the periaqueductal gray (PAG), among other regions (Lischinsky and Lin, 2020). Initially thought that each social behavior is associated with a specific pattern of activation in the overall SBN, recent evidence indicates that distinct sub-circuits are dedicated to specific social behaviors. For instance, sexual behavior, but not territorial aggression, is regulated by neurons in the BNSTpr that project to the mPOA, which then project to the ventral tegmental area (VTA) and the PAG (Bayless et al., 2023). By contrast, a core aggression circuit has been identified as a key regulator of aggression, which includes the BNSTpr, the postero-dorsal MeA (MeApd) and the ventro-lateral VMH (VMHvl) (Lischinsky and Lin, 2020).

Within these circuits, two brain regions are particularly important: the BNSTpr and the MeApd. Both regions receive olfactory and pheromonal information from the vomeronasal organ (VNO) via direct wiring from the accessory olfactory bulb (AOB) (Flanigan and Kash, 2022; Cádiz-Moretti et al., 2016; Dwyer et al., 2022). The BNSTpr and MeApd are highly interconnected and both send projections to the mPOA (Dong and Swanson, 2004; Keshavarzi et al., 2014; Kohl et al., 2018; Zhang et al., 2021), a key center for the modulation of sexual behavior, and to neurons located in the VMHvl, which control male-male aggression (Zha and Xu, 2021).

BNSTpr and MeApd also contain the highest density of aromatase-expressing neurons (Aro+) in the brain (Wu et al., 2009). These

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neurons are major regulators of the local production of estrogens in the brain through the aromatization of testosterone into estradiol (E2). E2 produces wide effects on neuronal physiology (Woolley, 2007) and regulates both sexual behavior and territorial aggression in male mice (Taziaux et al., 2007; Trainor et al., 2008). E2 also elicits organizational effects during development, promoting brain and behavior masculinization (Wu et al., 2009). In adults, E2 can induce rapid (few minutes) behavioral effects: systemic injections of E2 in male mice promotes sexual behavior and territorial aggression in 10 to 25 min (Taziaux et al., 2007; Trainor et al., 2008), whereas systemic aromatase inhibition inhibits sexual behavior within 10 to 20 min (Taziaux et al., 2007).

Aro + neurons have also been shown to directly regulate various social behaviors (Brann et al., 2022; Cornil et al., 2012). In naive male mice, inhibiting or ablating BNSTpr Aro + neurons drastically impairs sexual behavior and territorial aggression (Bayless et al., 2019), while inhibiting MeApd Aro + neurons impairs different forms of aggression, but not sexual behavior (Unger et al., 2015). However, the impact of social experience on the role played by these Aro + neurons for the display of sexual and aggressive behaviors remains largely unknown.

Sexual behavior and territorial aggression can be modified by experience (Sakata et al., 2022; Trainor et al., 2006). Sexually experienced mice or those with experience in male-male aggression show an increased motivation to initiate the corresponding behavior, as evidenced by a reduction in the latency to the first mount (Jean et al., 2021) or the first attack (Kwiatkowski et al., 2021). Sexual experience also causes motor improvements leading to a higher efficiency of the behavioral sequence with a reduced latency to the first intromission and a shorter time to reach ejaculation in sexually experienced animals compared to naive ones (Jean et al., 2017). Experience in male-male aggression also increases overall aggression in the Resident-Intruder paradigm (Kwiatkowski et al., 2021). However, it remains unclear whether the same neurons in the BNSTpr and MeApd that control sexual and aggressive behaviors are also involved in experience-dependent behavior modulation.

Experience-dependent improvement of sexual behavior is associated with a synaptic and molecular plasticity in the mPOA evidenced by an increase in mature dendritic spine density (Jean et al., 2021) and increased androgen receptor expression (Swaney et al., 2012). Similarly, a testosterone-dependent synaptic plasticity has been described in Esr1-positive neurons of the VMHvl after receiving inputs from the posterior amygdala (Stagkourakis et al., 2020). This mechanism promotes increased aggression in experienced animals, while its depression diminishes the behavioral effect of aggression training. Experience has also been shown to induce plastic changes at the sensory processing level. For example, *Arc*-expressing interneurons in the AOB undergo synaptic plasticity following aggression training, and chemogenetic inhibition of these neurons during training prevents an increase in aggression (Zuk et al., 2023). Yet, little is known about the effects of social experience on neurons downstream to the AOB, especially on aromatase neurons.

Here, we used a chemogenetic approach to inhibit BNSTpr Aro + neurons in socially-experienced male mice (i.e. trained for both aggression and sexual behavior) to determine differential contributions of this cell population in the display of sexual behavior and territorial aggression. We next used Egr1 as a reporter of neural activity to investigate the differential neural plasticity in the form of increased local activity driven by social experience in the context of sexual behavior and male-male aggression. Our results revealed that social experience triggers a behavior-specific plasticity in the VNO and MeApd. Thus, our data expose an experience-dependent link between sensory inputs in the accessory olfactory pathway with limbic regions containing Aro + neurons.

2. Materials and methods

2.1. Mice

We employed B6.129S(SJL)-*Cyp19a1*$^{tm2.1(cre)Shah}$/J Stock#: 027038; RRID: IMSR_JAX:027038 from the Jackson Laboratory (denoted as Aro^{cre} mice) that were crossed to 129sv wild type mice (Janvier Laboratories, France) to produce a mixed C57Bl/6 x 129sv background. To generate Aro^{Cre} Ribo^{Tag} mice, we crossed homozygotes Aro^{Cre} mice with RiboTag$^{loxP/loxP}$ mice (B6N.129(Cg)-Rpl22$^{tm1.1Psam}$/J from the Jackson laboratory Stock#: 011029; RRID: IMSR_JAX: 011029 (Sanz et al., 2019). Adult C57Bl/6 males and ovariectomized receptive females (see supplementary material) were used as stimulus animals (Janvier). Mice were housed under standard 12 h light/dark cycles with regular chow diet and water ad libitum.

2.2. Social experience protocol

2.2.1. Sexual behavior

Sexually naive male mice (>8 weeks) were individually housed from 4 days before training. The training began when an unfamiliar ovariectomized receptive female was introduced in their home cage overnight (bedding not changed for at least 4 days). Ejaculation was confirmed by video recordings and latency to ejaculation was scored. From this time point, mice remained individually housed until euthanasia. A second session was performed 14 days later with the introduction of a new unfamiliar receptive female in their home cage overnight. Only males that were successful in the two sessions, with a significant reduction of the latency to ejaculation (Fig. S1, left) were kept for the following experiments.

2.2.2. Territorial aggression

Sexually experienced males (as described above) were trained for territorial aggression by 3 consecutive Resident-Intruder tests (1 per day) (Chamero et al., 2007; Kwiatkowski et al., 2021). Briefly, an unfamiliar and gonadally intact naive male was introduced into the resident home cage (bedding not changed for at least 4 days) for 10 min and latency to the first attack was scored. Aggressive behavior was defined as lunging, biting, chasing, tail rattling, wrestling and kicking. If aggression was too intense (i.e. excessive physical damage and presence of blood in the intruder), the test was interrupted. Residents were not exposed twice to the same intruder. Intruders were only exposed to one resident during each behavioral session (i.e. each day). However, to guarantee that intruders were always unfamiliar to the residents, each intruder was exposed to a different resident across different behavioral sessions.

2.3. Stereotaxic injections

2.3.1. Surgeries

BNSTpr and mPOA coordinates were experimentally determined considering a ten-degree angle (distance from bregma: **BNSTpr** - AP: -0.18; ML: ±1.28; DV: 5.07 and 4.07 / **mPOA** – AP: 0; ML: ±1.3; DV: −5.4 and − 5,1). All injections were performed at a rate of 100 nL/min with a 5 μL Hamilton Neurosyringe (33 gauge, not beveled). To target the whole BNSTpr in the dorso-ventral axis, two consecutive injections of 250 nL of virus placed 1 mm apart were used. Mice were thus injected bilaterally with 500 nL of virus. In the mPOA, 150 nL were injected more ventrally and 300 μm above, representing a total of 300 nL per hemisphere. After injection, the needle was kept in place for two minutes and slowly removed. Mice were allowed to recover for two weeks before starting behavioral experiments. For details about the whole surgery, see supplementary material.

2.3.2. Viruses

For the BNSTpr chemogenetic experiments, we used an AAV1/2-hSyn-flx-hM4Di-mCherry-flx to inhibit aromatase neurons (5.9*10^12

viral genomes/mL, *reference* # V-84-1) or an AAV1/2-hSyn-flx-mCherry-flx as a control (5.4*10^12 viral genomes/mL, *reference* # V-116-1). These viruses were purchased from the Viral Vector Facility of the University of Zurich (Switzerland). Undiluted stock solutions were bilaterally injected in the BNSTpr as described above. For mPOA retrograde tracing experiments, we used a canine adenovirus 2 (CAV2) hSyn1-flx-mCherry-flx (10*10^12 pp./mL) purchased from EJ Kremer in the Plateforme de Vectorologie de Montpellier, France (PVM) (del Rio et al., 2019). On the day of the injection, a viral aliquot was thawed on ice and diluted just before injection to a final concentration of 1*10^9/150 nL in sterile saline.

2.3.3. Chemogenetics

Individually housed and trained Aro^{Cre} males were tested in their home cage for a 10 min Resident-Intruder aggression test on day 38, and for a 30 min sexual behavior test on day 41 (Fig. 1b). All animals were habituated to the whole behavioral protocol (handling, injections, red light) during 3 days. We randomly assigned animals to receive IP injections of either CNO (3.5 mg/kg) or sterile saline 30 min before the test. The timing and dose of CNO injection were chosen to ensure CNO concentrations in the brain higher than its EC_{50} for hM4Di (Jendryka et al., 2019). CNO (Selleck) was dissolved in sterile saline at 12.5 mg/mL, aliquoted and stored at −20 °C. On each test day, a CNO aliquot was thawed and diluted to 0.35 mg/mL in sterile saline. On a given week, the assigned treatment was the same on day 1 (e.g. D38) and day 4 (e.g. D41) (CNO or saline). The experiment was repeated the following week and the treatment was switched (animals that previously received CNO received then sterile saline on day 1 (e.g. D45) and 4 (e.g. D48) and vice versa). This whole 2-week experiment was then repeated. Bedding was changed 1 week before the start of the whole experiment. To keep the animals in a standardized bedding state during the whole experiment (1 month), 1/3 of the soiled bedding was replaced by fresh one following each territorial aggression test (once a week). Behaviors were video recorded and scored manually by a blind annotator using Boris software (Friard and Gamba, 2016).

2.3.4. Histology

Fixed and frozen coronal brain slices were obtained as described in supplementary material. For chemogenetic experiments, only mice densely expressing mCherry bilaterally along the dorso-ventral axis of the BNSTpr were analyzed. For retrograde tracing experiments, mice were analyzed when the injection site was in the mPOA and mCherry expression was observed in the target regions (BNSTpr and MeApd). Immunolabeling and in situ hybridization were performed as described in supplementary material. 2–3 brain sections (30 μm each) per animal were imaged using a Zeiss LSM780 confocal microscope and for each region of interest, 3–4 z-series optical sections were collected. All confocal images were z-normalized using a custom Python script (G-Node DOI: 10.12751/g-node.cs3tdm) and cells in each region of interest (ROI) was classified as described in supplementary material.

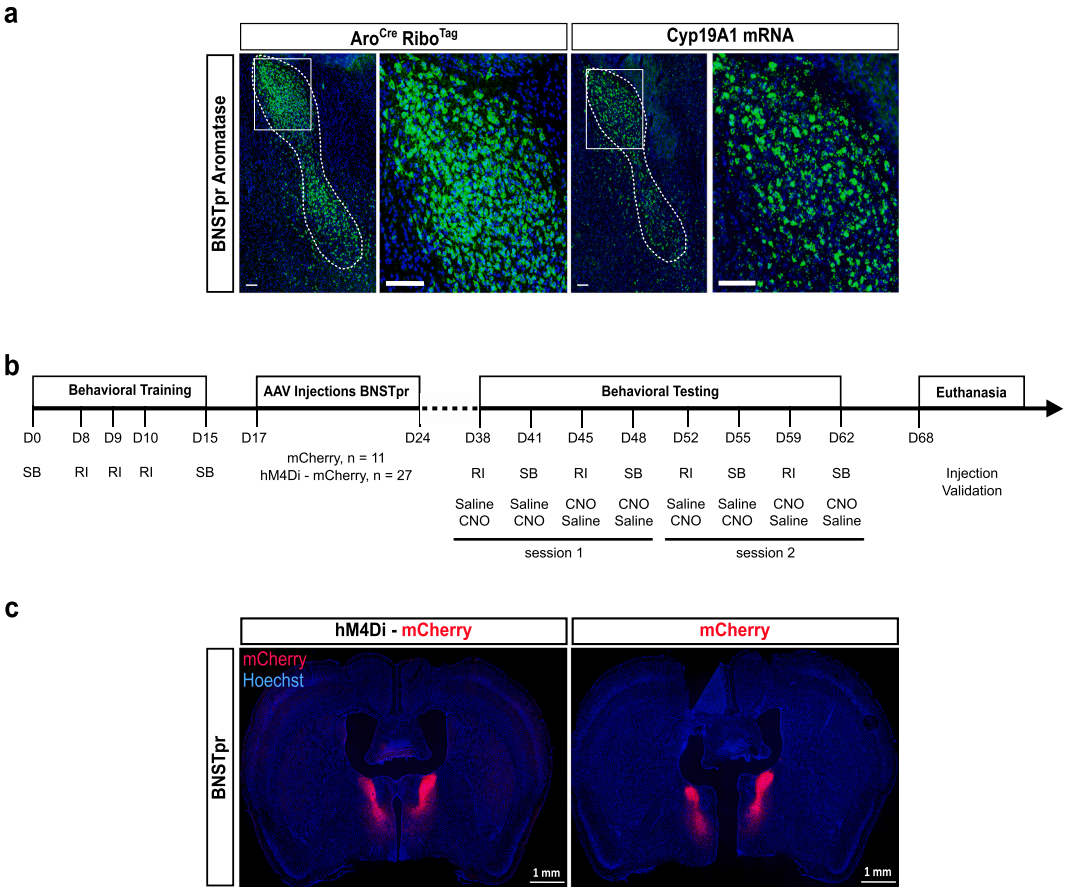

Fig. 1. Aromatase distribution in the BNSTpr and chemogenetic experimental timeline. **(a)** left, Aromatase positive (Aro+) cells (green) in the BNSTpr visualized through tag immunolabeling in Aro^{Cre}Ribo^{Tag} mice and right, in situ hybridization against aromatase (*Cyp19a1* gene). **(b)** Schematic representation of the experimental timeline used for the BNSTpr Aro + chemogenetic inhibition in experienced males. SB: Sexual Behavior, RI: Resident-Intruder, D: Day. **(c)** Representative images of bilateral viral injection sites (red staining) in the BNSTpr of animals injected with AAVs coding for hM4Di-mCherry (left) or mCherry (right). Blue, Hoechst nuclear staining. Scale bars, 1 mm.

2.4. Behaviors

2.4.1. Territorial aggression

To evaluate territorial aggression, 4 behavioral parameters were scored: (1) the number of attacks (i.e. each attack bout containing either chasing, biting, wrestling or boxing was counted as one attack), (2) the time spent in aggression (i.e. the total duration spent chasing, biting, wrestling or boxing), (3) the time spent tail rattling (i.e. the total duration of all tail rattling events) and (4) the latency to the first attack (i.e. the time interval between the introduction of the intruder in the resident's cage and the first attack). The proportion of males showing at least one attack was also computed.

2.4.2. Sexual behavior

To evaluate sexual behavior, 4 behavioral parameters were scored: (1) the time spent in anogenital investigation (i.e. when the male's nose is in close contact with the female's anogenital area and actively investigating it), (2) the latency to the first mount (i.e. the time interval between the introduction of the female in the male's cage and the first copulatory event (mount with or without intromission), (3) the time spent mounting (with and without intromission) and (4) the latency to ejaculation (i.e. the time interval between the introduction of the female in the male's cage and the ejaculation). The proportion of males mounting, intromitting and ejaculating was also computed.

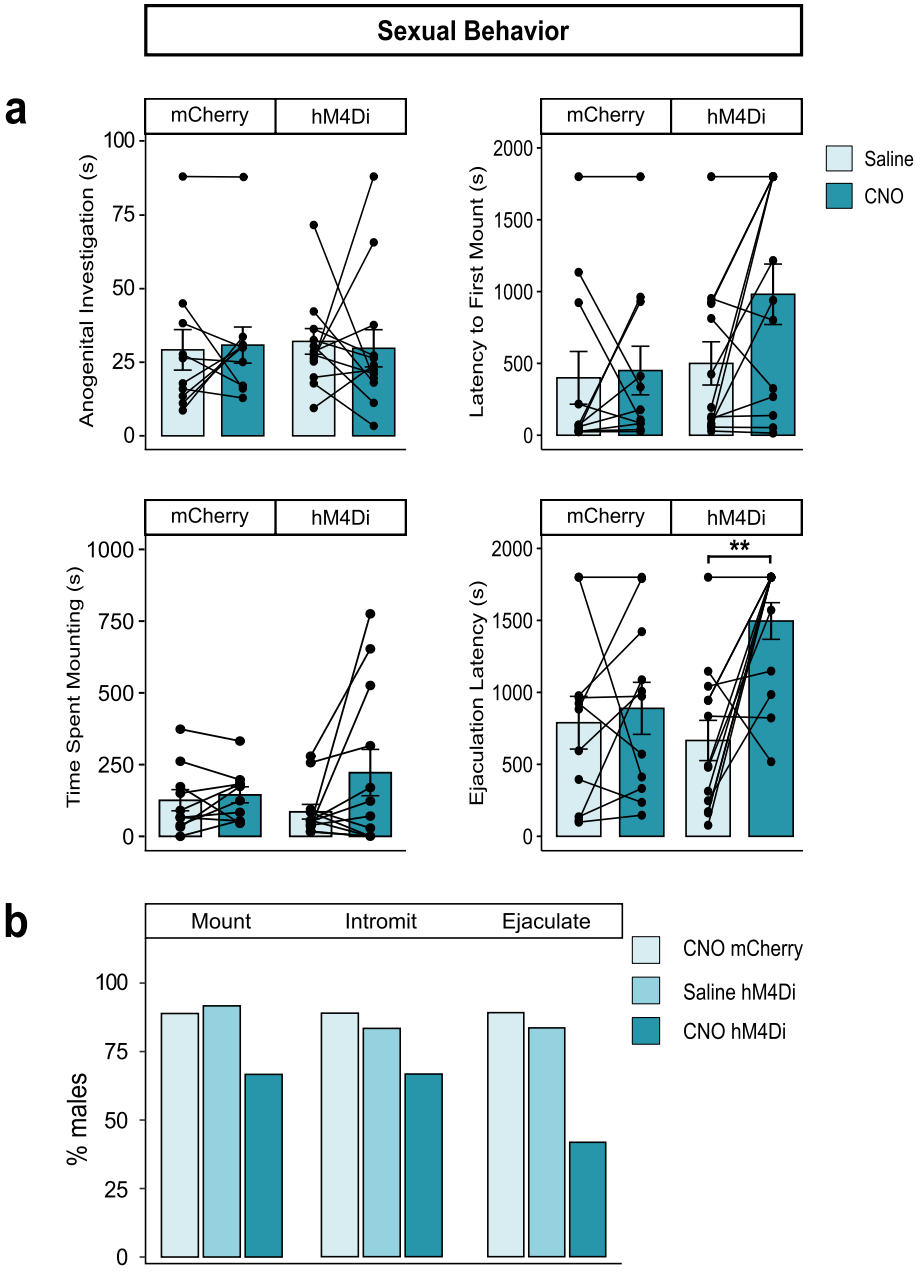

Fig. 2. A high proportion of socially experienced male mice retain functional sexual behavior after BNSTpr Aro + cells inhibition. **(a)** Parameters of sexual behavior in hM4Di ($n = 9$–13) and mCherry ($n = 10$–11) animals after saline or CNO injection. Each point is the mean over two behavioral sessions for each individual mouse. Bars depicts the mean of each group together with the Standard Error of the Mean (SEM) as error bars. For each plot, a permutation based linear mixed model has been performed to test for an interaction effect between group and treatment. When interaction resulted in a p value <0.05, a paired unilateral Wilcoxon permutation test was performed in the hM4Di group to compare saline and CNO treatment. Bonferroni correction for 4 comparisons was applied to p-values; ** $p \leq 0.01$. **(b)** Proportion of males mounting, intromitting and ejaculating. Data were analyzed with paired one-sided exact McNemar tests and p values adjusted for three comparisons.

2.4.3. Statistical analysis and software

Statistical analyses were performed using the statistical software R version 4.2.3. Since most of our data did not meet normality and homoscedasticity requirements, we chose to perform permutation-based statistical tests for the whole data analysis (Berry et al., 2002; Hothorn et al., 2008; Anderson, 2001). Effect sizes for pairwise comparisons are reported as Cohen's *d* or Hedge's *g* where appropriate (Lakens, 2013). The significance threshold (alpha) was set to 0.05. Sample size (N), *p*-values, effect sizes estimates and the specific statistical test performed for each experiment are indicated in the main text and figure legends, and recapitulated in the Supplementary Table 1.

2.4.4. Training

Latency to ejaculation and latency to the first attack were evaluated before and after the training to confirm that trained animals are quicker to reach ejaculation and to attack intruders (Fig. S1). Behavioral data were compared using a unilateral Wilcoxon test with permutations.

2.4.5. Chemogenetic

Behavioral data was scored with Boris and analyzed using a custom R

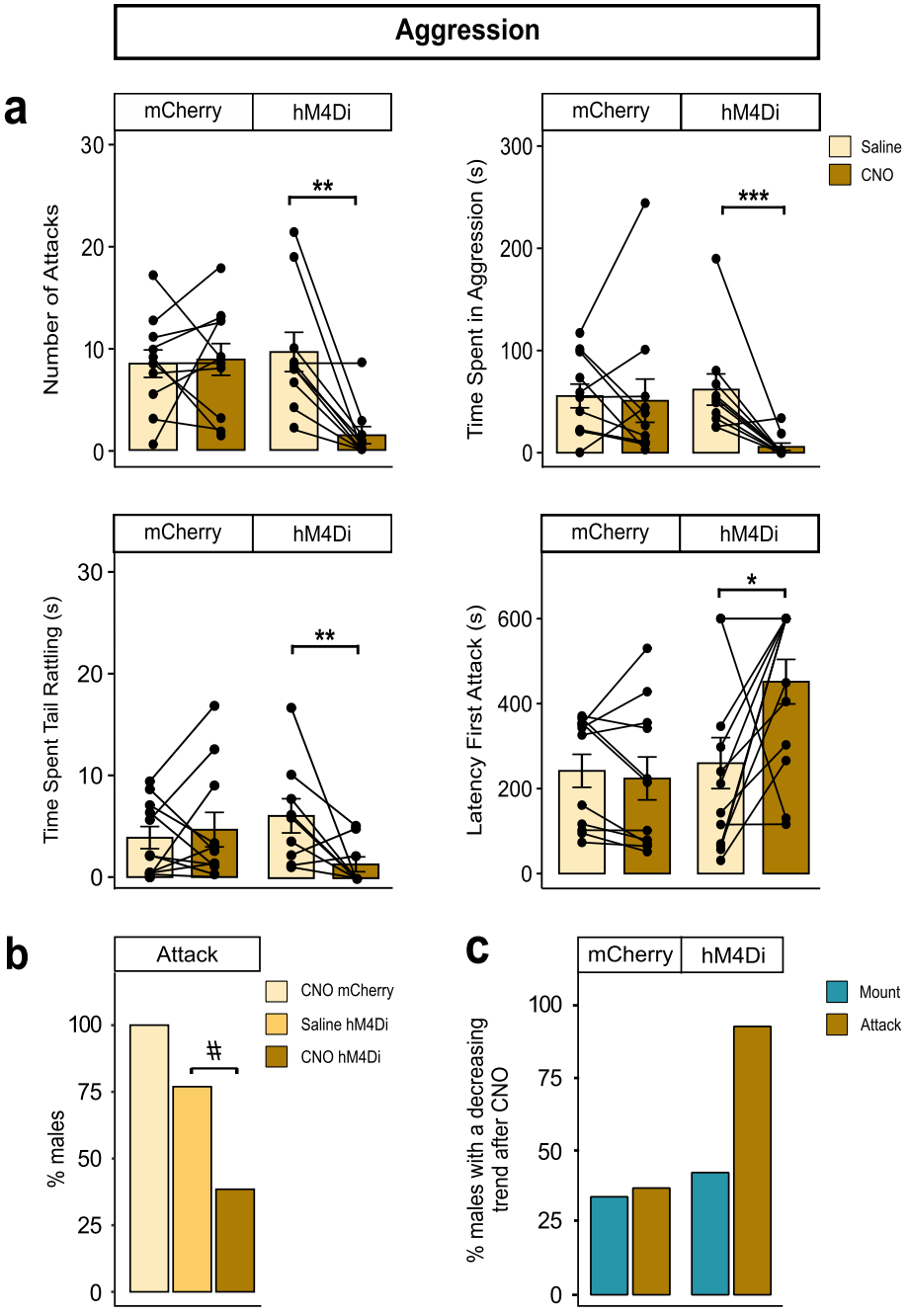

Fig. 3. BNSTpr Aro + cells in socially experienced male mice are necessary for the display of male-male aggression. **(a)** Parameters of aggression in hM4Di (n = 9–13) and mCherry (n = 10–11) animals after saline or CNO injection. Each point is the mean over two behavioral sessions for each individual mouse. Bars depicts the mean of each group together with the SEM (error bars). For each plot a permutation based linear mixed model has been performed to test for an interaction effect between group and treatment. When interaction resulted in a p value <0.05, a paired unilateral Wilcoxon permutation test was performed in the hM4Di group to compare saline and CNO treatment. Bonferroni correction for 4 comparisons was applied to *p*-values; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p < 0.001$. **(b)** Proportion of males executing territorial aggression. Data were analyzed with paired one-sided exact McNemar tests and the *p* value adjusted for 1 comparison (i.e. no adjustment); # $p = 0.06$. **(c)** Proportion of males showing a decreasing trend in mount number (mount) or attack number (attack) after CNO injection in mCherry and hM4Di animals.

script. For latency values, animals not displaying the behavior were given a value of the total duration of the test (i.e., aggression = 600 s and sexual behavior = 1800s). Individual values represent the mean of all behavioral parameters (except for proportions) over two trials for each individual mouse. A linear mixed model with permutations was used to test a two-factor model including the group (hM4Di vs. mCherry) and the treatment (CNO vs. Saline). When the interaction was significant, we performed a planned comparison testing the effect of CNO in the hM4Di group with a unilateral paired Wilcoxon test with permutations. Since we tested 4 behavioral parameters, *p*-values were corrected for 4 comparisons using the Bonferroni method. For proportions, only the second session is presented in Figs. 2b and 3b and results from hM4Di animals receiving CNO (treated) vs. Saline (controls) were compared using a unilateral exact McNemar test. Given that 3 variables were studied for sexual behavior (mount, intromission, ejaculation), p-values were corrected for 3 comparisons using the Bonferroni method. Since only one parameter was analyzed for aggression (attack), the p-value was not corrected.

2.5. Confocal images

Proportions of the different cell types were computed for each slice. Plots represent the mean proportion in each animal. Outliers were identified using the IQR method and removed for further analyses. We performed unilateral two-sample Fisher-Pitman permutation tests for the comparison of the total Egr1 cell proportions between naive and experienced animals, for each behavior. Given that we tested 9 brain regions, all p-values were adjusted for multiple comparisons using the Bonferroni method. For the retrograde tracing analysis, we used the same statistical test, but p-values were corrected for 4 comparisons (2 regions × 2 cell classes). The VNO was analyzed separately using a unilateral two-sample Fisher-Pitman permutation test.

3. Results

3.1. BNSTpr Aro + cells are necessary for the display of male-male aggression, but not for sexual behavior, in socially experienced male mice

To evaluate whether Aro + neurons are required in socially-experienced animals, we first crossed Aro^{Cre} males with a mouse line expressing the Cre-dependent reporter Ribotag (Sanz et al., 2019) and visualized Cre + cells in the BNSTpr. We observed a dense population of Tag+ cells present in the BNSTpr after immunodetection (Fig. 1a, left). Aromatase (*Cyp19a1*) gene mRNA labeling via in situ hybridization (Fig. 1a, right) showed a similar cell distribution in the BNSTpr, indicating that Cre recombinase is largely expressed in Aro + neurons in the Aro^{Cre} mouse. We then performed a 3-week training of naive Aro^{Cre} males for sexual behavior and territorial aggression (see methods) (Fig. 1b and Fig. S1). Next, we injected the experienced males bilaterally in the BNSTpr with a Cre-dependent adeno associated virus (AAV) encoding for hM4Di-mCherry (Fig. 1c). Then, we used a chemogenetic approach (Alexander et al., 2009) to inhibit Aro + cells by injecting clozapine n-oxide (CNO) to socially experienced Aro^{Cre} animals prior to behavior testing. We hereafter refer to hM4Di injected animals as "treated" when they received CNO before behaviors, and as "controls" when they received saline before behaviors. To control for any CNO side effect, we used CNO-treated Aro^{Cre} animals expressing mCherry reporter alone, which we refer as "mCherry controls" (Fig. 1b-c).

Behavior scoring showed that chemogenetic inhibition of BNSTpr Aro + cells had a limited impact on sexual behavior when treated animals are compared to controls (Fig. 2a-b). The proportion of treated males mounting and intromitting was slightly lower compared to controls (61 % vs 84 % and 61 % vs 76 %, respectively), although this difference was not statistically significant ($p.adj$ = 0.37, 0.93, respectively). 38 % of treated animals ejaculated, compared to 76 % of controls, but this difference was not significant ($p.adj$ = 0.18) (Fig. 2b). These results show that a high proportion of treated animals still displayed full sexual behavior. We further analyzed other behavioral parameters during the motivational phase such as the time spent in anogenital investigation ($Z = 1.15$, $p.adj = 0.49$, Cohen's $d = 0.11$) and the latency to the first mount ($Z = -1.54$, $p.adj = 0.24$, Cohen's $d = 0.73$), but did not find significant differences between controls and treated groups (Fig. 2a). Similarly, these animals displayed a comparable mounting time during the copulatory phase ($Z = -0.34$, $p.adj = 1$, Cohen's $d = 0.65$; Fig. 2a), although we observed a significant increase in the latency to ejaculation in treated vs. control animals ($Z = -3.32$, $p.adj = 0.0017$, Cohen's $d = 1.72$; Fig. 2a), indicating an incomplete inhibition of sexual behavior.

By contrast, chemogenetic inhibition of BNSTpr Aro + neurons of socially experienced males showed a higher impact on territorial aggression. The number of attacks ($Z = 3.30$, $p.adj = 0.0019$, Cohen's $d = 1.72$), the attack duration ($Z = 3.58$, $p.adj = 0.0006$, Cohen's $d = 1.59$) and the time spent tail rattling ($Z = 2.74$, $p.adj = 0.012$, Cohen's $d = 1.23$) were 4 to 10-fold lower in treated animals, and they took longer to initiate the first attack compared to controls ($Z = -2.27$, $p.adj = 0.046$, Cohen's $d = 0.94$; Fig. 3a). The proportion of males attacking at least once was reduced from 76 % in treated to 38 % in control animals ($p.adj = 0.062$) (Fig. 3b). We next examined the percentage of males that displayed a decrease in mounting and attack behavior after CNO injection, and found that 90 % of the treated animals attacked less, while only 41 % showed less mounting after CNO, a value similar to mCherry controls (Fig. 3c). Together, these results indicate that chemogenetic inhibition of BNSTpr Aro + neurons in socially-experienced male mice causes a robust reduction of territorial aggression, while only causing limited impact on sexual behavior.

3.2. Social experience triggers a behavior-specific activity increase in the MeApd

We hypothesized that the incomplete inhibition of sexual behavior in socially experienced males may result from experience-induced plasticity. This may be denoted in the form of higher BNSTpr activity, which may render inhibition less effective and/or the recruitment of an alternative/complementary olfactory pathway, bypassing Aro + BSNTpr neurons. To test whether an increase in activity in the BSNTpr occur after experience, we performed immunohistochemistry (IHC) for the immediate early gene *Egr1* as a proxy of neural activity in coronal brain slices of naive versus experienced animals after sexual behavior or territorial aggression (Figs. 4a and 5). We found a 4.3-fold significant increase of Egr1+ cells in the BNSTpr of socially experienced animals after territorial aggression ($Z = -3.32$, $p.adj = 0.004$, Hedge's $g = 4.39$; Fig. 5). A similar tendency (4.4-fold Egr1+ cell increase) was found after sexual behavior, but the difference was not statistically significant ($Z = -2.24$, $p.adj = 0.11$, Hedge's $g = 1.48$; Fig. 5).

We also quantified the number of Egr1+ cells in the MeApd (Fig. 6), a region containing a high density of Aro + neurons that is directly innervated by the vomeronasal system (Dwyer et al., 2022). Following territorial aggression, the proportion of Egr1+ cells in this region between naive and experienced animals was not significantly different ($Z = -2.16$, $p.adj = 0.13$, Hedge's $g = 1.35$; Fig. 6). By contrast, we observed a much larger 7.5-fold increase in the mean proportion of Egr1+ cells after sexual behavior in experienced (10 %) vs. naive (1.3 %) animals ($Z = -2.94$, $p.adj = 0.014$, Hedge's $g = 2.36$; Fig. 6). These results indicate that social experience causes a robust increase in the number of Egr1+ cells in the MeApd following sexual behavior, but not after territorial aggression.

3.3. Social experience triggers a behavior-specific activity increase in the VNO

Sexual behavior and territorial aggression in rodents are highly dependent on olfactory signals detected by VNO sensory neurons (VSNs)

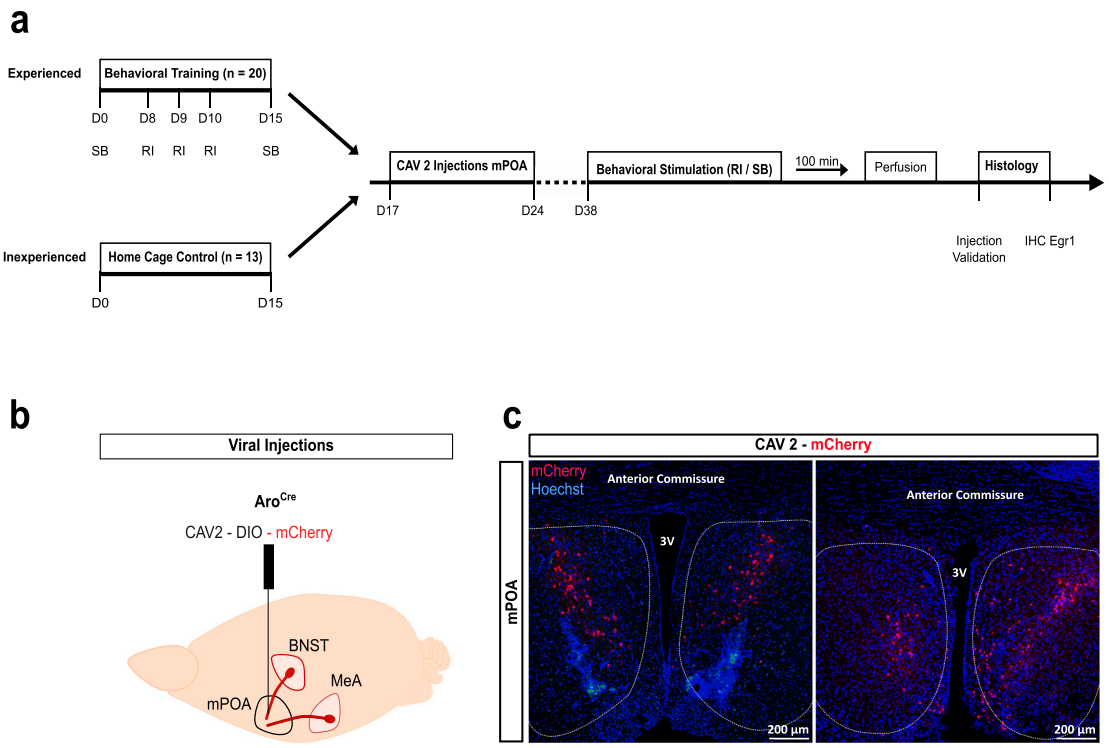

Fig. 4. Egr1 immunolabeling and retrograde tracing timeline. **(a)** Schematic representation of the experimental timeline used for immunodetection of Egr1 in inexperienced (naive) vs. experienced animals and subsequent retrograde tracing. SB: Sexual Behavior, RI: Resident-Intruder, D: Day, IHC: Immunohistochemistry. **(b)** Aro^{Cre} males ($N = 33$) were injected in the mPOA with the retrograde tracer Canine Adenovirus 2 (CAV2) coding for a floxed mCherry. Aro + cells projecting to the mPOA are then tagged with mCherry and co-labeled with Egr1. **(c)** Representative images of bilateral viral injection sites in the mPOA of two different animals injected with mCherry-CAV2 (red staining). Nuclei are counterstained with Hoechst (blue). 3 V, third ventricle. Scale bars, 200 µM.

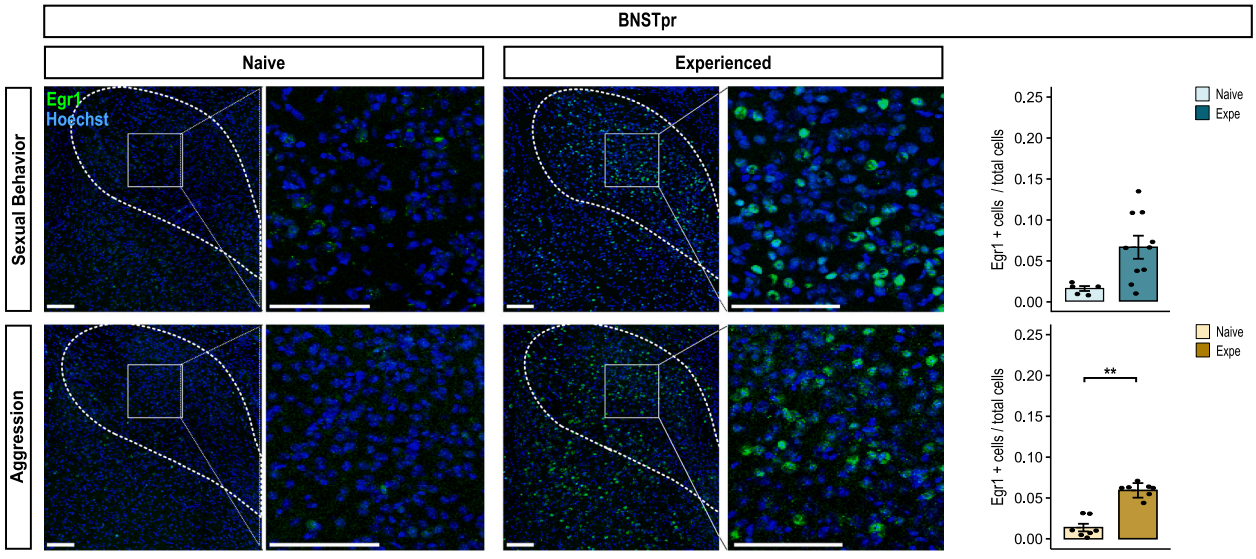

Fig. 5. Social experience tends to trigger an activity increase in the BNSTpr following aggression and sexual behavior. Left, representative images of Egr1 immunolabeling (green) in the BNSTpr in naive ($n = 5$–7) and experienced animals ($n = 7$–11). Nuclei were labeled with Hoechst (blue). Scale bars, 100 µM. Right, proportion of Egr1-positive cells per slice after sexual behavior (top) or territorial aggression (bottom). Each point represents one animal. Error bars depicts SEM. Naive and experienced animals were compared using a unilateral two-sample Fisher-Pitman permutation test and *p*-values adjusted for 9 comparisons (9 brain regions in combination with Figs. 6, 7 and 8); ** $p \leq 0.01$.

(Stowers et al., 2002; Leypold et al., 2002; Chamero et al., 2007, 2011). The BSNTpr and MeApd receive indirect inputs from the VNO via the AOB (Dwyer et al., 2022; Holy, 2018). We thus explored whether the higher activity observed in the MeApd after social experience is accompanied by a higher activity in the VNO. We performed IHC staining for Egr1 on coronal VNO slices in naive vs. experienced animals after sexual behavior or territorial aggression (Fig. 7). We found that experienced animals showed a significantly higher (1.9-fold) number of Egr1+ cells in the VNO after sexual behavior ($Z = -2.0028$, $p = 0.022$, Hedge's $g = 1.63$, Fig. 7, top), but not after territorial aggression ($Z =$

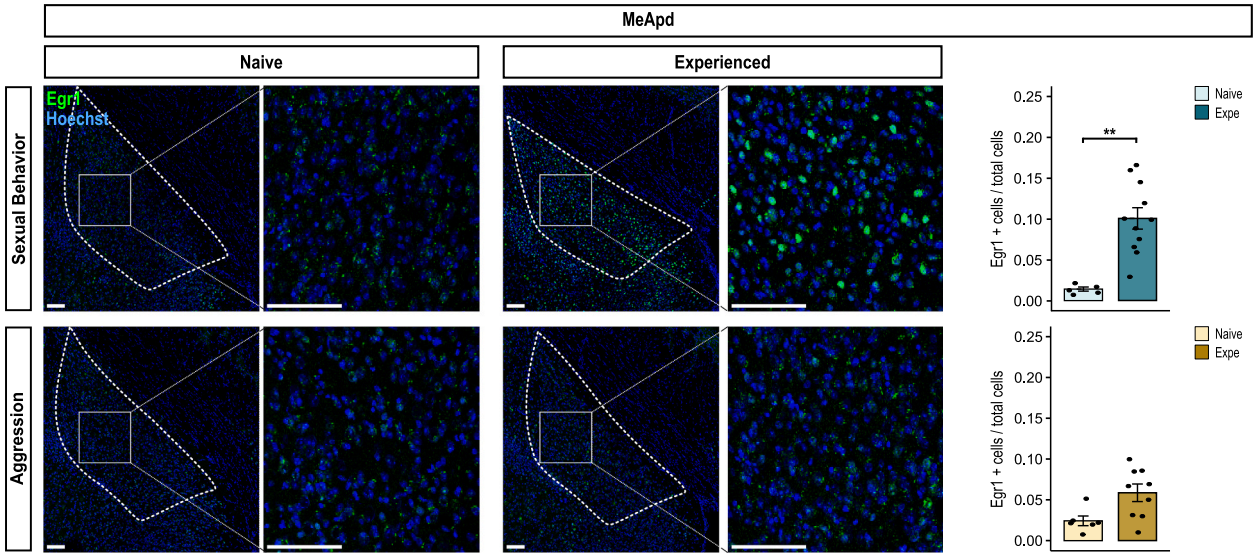

Fig. 6. Social experience triggers a behavior-specific activity increase in the MeApd. Left, representative images of Egr1 immunolabeling (green) in the MeApd in naive (n = 5–7) and experienced animals (n = 7–11). Nuclei were labeled with Hoechst (blue). Scale bars, 100 μM. Right, proportion of Egr1-positive cells per slice after sexual behavior (top) or territorial aggression (bottom). Each point represents one animal. Error bars depicts SEM. Naive and experienced animals were compared using a unilateral two-sample Fisher-Pitman permutation test and *p*-values adjusted for 9 comparisons (9 brain regions in combination with Figs. 6, 7 and 8); ** $p \leq 0.01$.

−1.43, $p = 0.07$, Hedge's $g = 0.99$; Fig. 7, bottom). These data suggest that social experience causes a behavior-specific neural plasticity in the VNO that result in higher sensory cell activation rate.

3.4. *Social experience triggers a behavior-specific plasticity in MeApd Aro + neurons projecting to the mPOA*

BNSTpr and MeApd neurons innervate hypothalamic regions, such as the VMH and mPOA, that play an important role for the display of aggressive and sexual behaviors. Notably, the mPOA receives direct projections from the BNSTpr and the MeApd (Dong and Swanson, 2004; Keshavarzi et al., 2014), and mPOA lesions drastically inhibit sexual behavior (Liu et al., 1997). To better understand how social experience modulates hypothalamic activity, we screened for Egr1 activity in six hypothalamic regions: mPOA, Arcuate nucleus (Arc), dorsomedial hypothalamic nucleus (DMH), VMHvl, central VMH (VMHc), dorsomedial VMH (VMHdm) (Fig. 8). Among all these regions, only the mPOA experienced a significant increase in Egr1+ cells. We observed a 3.1–4-fold increase of Egr1+ cells after experience following both sexual behavior ($Z = -2.58$, $p.adj = 0.044$, Hedge's $g = 1.8$) and territorial aggression ($Z = -2.80$, $p.adj = 0.022$, Hedge's $g = 5.15$), respectively (Fig. 8).

Given the high density of Aro + neurons in the MeApd, we reasoned that MeApd Aro + cells projecting to the mPOA may be specifically activated in experienced mice following sexual behavior. To test this possibility, we injected a Canine Adenovirus 2 (CAV2) coding a floxed mCherry in the mPOA of Aro^{Cre} males to retrogradely trace Aro + neurons projecting to the mPOA (Fig. 4b-c). We then performed IHC staining for Egr1 in naive and experienced animals after sexual behavior or territorial aggression and quantified the proportion of cells co-expressing mCherry and Egr1 in the BNSTpr and MeApd. We observed a 3.5–4.9 -fold experience-dependent increase of Egr1+/mCherry+ neurons in the BNSTpr following both sexual and aggressive behaviors (sex: $Z = -2.21$, $p.adj = 0.053$, Hedge's $g = 1.45$; aggression: $Z = -2.24$, $p.adj = 0.049$, Hedge's $g = 1.57$; Fig. 9). A similar 3–3.6-fold increase was observed for Egr1+/mCherry- cells (sex: $Z = -2.04$, $p.adj = 0.08$, Hedge's $g = 1.2$; aggression: $Z = -2.48$, $p.adj = 0.025$, Hedge's $g = 1.66$; Fig. 9), indicating a global increase in Egr1 activity in the BNSTpr following the two behaviors. In the MeApd, the proportions of both cell populations showed a significant 5–7-fold increase after sexual behavior (mCherry+: $Z = -2.79$, $p.adj = 0.01$, Hedge's $g = 2.01$; mCherry-: $Z = -2.89$, $p.adj = 0.007$, Hedge's $g = 2.17$; Fig. 10). Importantly, and in contrast to the BNSTpr, territorial aggression did not induce any significant increase of Egr1+ mCherry+ or - cells in experienced animals (mCherry+: $Z = -0.68$, $p.adj = 0.98$, Hedge's $g = 0.34$; mCherry -: $Z = -1.76$, $p.adj = 0.15$, Hedge's $g = 1$; Fig. 10). These results indicate that a fraction of Aro + neurons in BNSTpr and MeApd that project to the mPOA, is activated during social behaviors. Experienced animals show a higher proportion of activity in these (and other) cells in the MeApd after sexual behavior, but not after territorial aggression, suggesting that the MeApd is subjected to a functional plasticity specifically after sexual experience.

4. Discussion

Although sexual behavior and territorial aggression can be initiated without prior experience, both the motivation to engage in these behaviors and the performance increases dramatically in experimented animals (Jean et al., 2021; Kwiatkowski et al., 2021). Here we show that chemogenetic inhibition of BNSTpr Aro + cells in socially experienced male mice drastically reduces territorial aggression. These results contrast with sexual behavior performance, which is barely affected by the inhibition. Among the four behavioral parameters tested for sexual behavior, only latency to ejaculation was significantly altered after BNSTpr Aro + inhibition (Fig. 2a, bottom right). Notably, this effect is completely caused by animals that do not ejaculate. Therefore, BNSTpr Aro + inhibition does not seem to alter the latency to ejaculation in animals that ejaculate, but rather indicates that inhibition reduces the proportion of males able to ejaculate (Fig. 2b, right). This suggests that some animals were resistant (no observed alteration in sexual behavior) to BNSTpr Aro + inhibition, whereas others were sensitive, based on their inability to ejaculate within the time of the test. Nevertheless, this effect on sexual behavior is minor compared to what has been previously shown in naive animals (Bayless et al., 2019). One relevant aspect of our chemogenetic results lies in the behavior-specificity of the observed phenomenon. Territorial aggression improves in experienced animals (Fig. S1) (Kwiatkowski et al., 2021), and BNSTpr neurons (including Aro+) play a crucial role in the display of aggression in socially

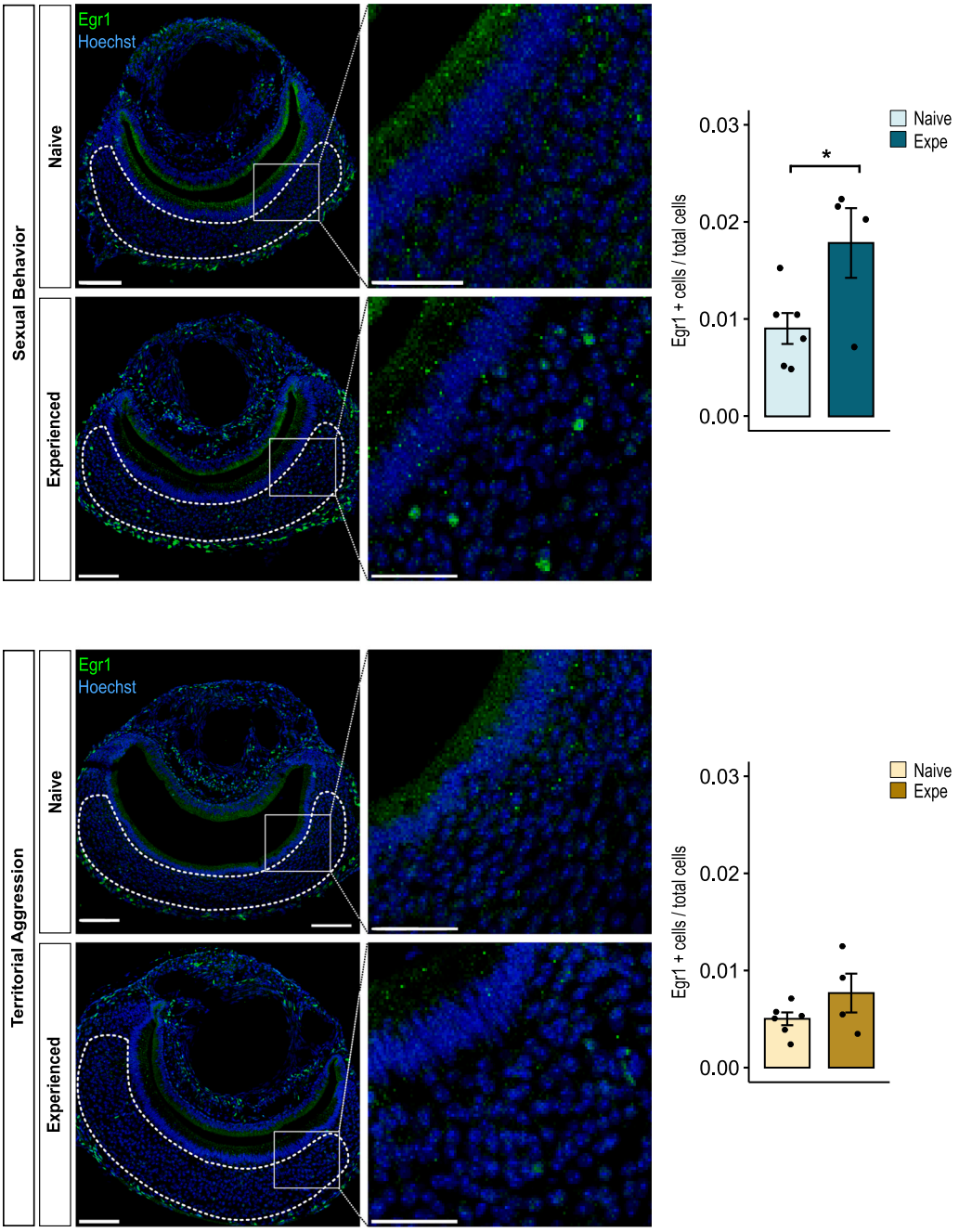

Fig. 7. Social experience triggers a behavior-specific activity increase in the VNO. Left, Coronal VNO sections stained with an anti-Egr1 antibody (green) and Hoechst (blue) after sexual behavior (top) or territorial aggression (bottom) in naive ($n = 6$) vs. experienced animals ($n = 4$). Dashed lines show the VNO area subject to Egr1 quantification. Scale bars, 100 μM. Right, proportion of Egr1 positive cells per section (3 sections/animal) after sexual behavior (top) or territorial aggression (bottom). Error bars depicts SEM. Groups were compared with a unilateral two-sample Fisher-Pitman permutation test; * $p < 0.05$.

experienced animals (Nordman et al., 2020). We observed that BNSTpr Aro + inhibition largely impairs aggression, but not sexual behavior, suggesting that these two behaviors are controlled by different Aro + neuron subsets after social experience. In this scenario, neural plasticity in one specific Aro + subpopulation may explain the mechanisms behind this differential regulation. Alternatively, social experience may induce the recruitment of parallel redundant circuits bypassing the BNSTpr, such as direct olfactory inputs to the MeApd. We thus screened the expression of Egr1 in several brain regions, including in the SBN, to evaluate the potential recruitment of neurons from other regions after social experience. For most of these regions (VMH, Arc, DMH, MeApv), we did not find any significant difference in the number of Egr1 cells after social experience (Fig. 8).

However, we found a sexual behavior-specific plasticity in the form of higher number of Egr1+ cells in the MeApd after social experience. While we also observe an increase of Egr1+ cells in the BNSTpr following social experience, this phenomenon occurred following both behaviors and it is thus unlikely to explain the behavior-specificity of the chemogenetic inhibition. Consistent with our results, a study using in vivo calcium imaging of the MeA showed a higher number of neurons recruited specifically when presented with a female congener in sexually experienced compared to naive male mice (Li et al., 2017). Furthermore, we show that the increase of Egr1 expression in experienced animals affects a subpopulation of MeApd Aro + cells which project to the

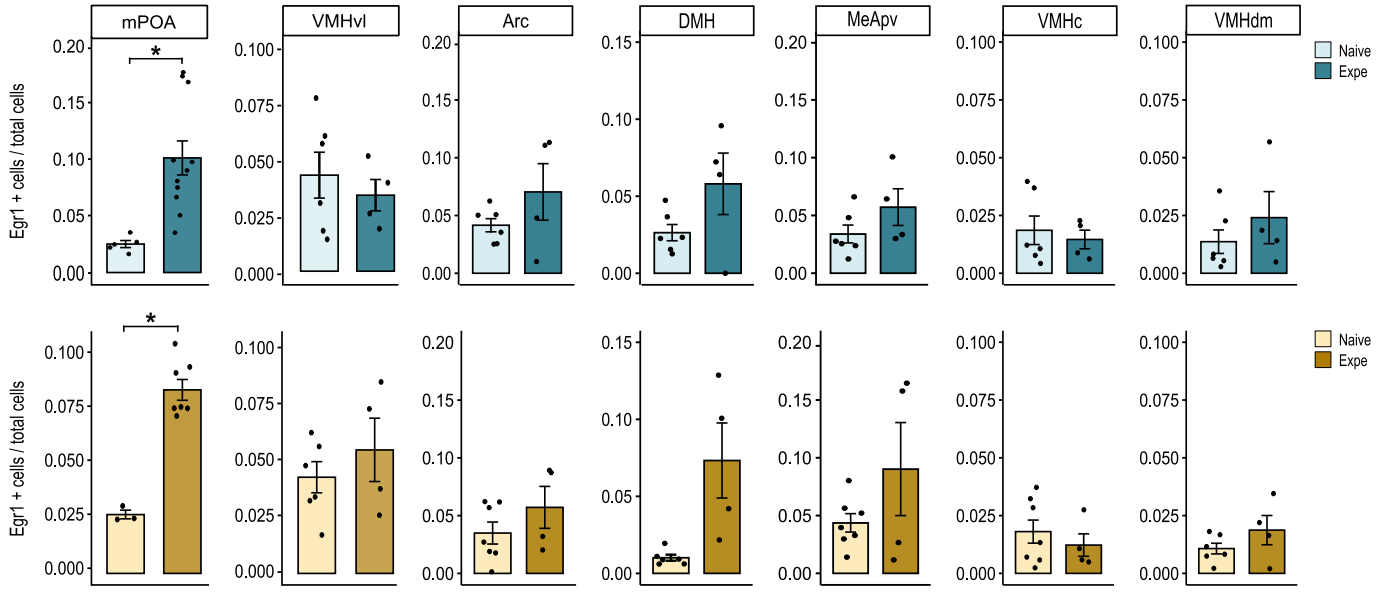

Fig. 8. Proportion of Egr1+ cells in other hypothalamic and amygdalar regions. Quantification of Egr1 immunostaining in different brain structures (2–3 slices per animal) after sexual behavior (top) or territorial aggression (bottom) in naive (n = 5–7) vs experienced animals (n = 4–11); * $p \leq 0.05$. Media preoptic area (mPOA), Arcuate nucleus (Arc), dorsomedial hypothalamic nucleus (DMH), ventrolateral VMH (VMHvl), central VMH (VMHc), dorsomedial VMH (VMHdm).

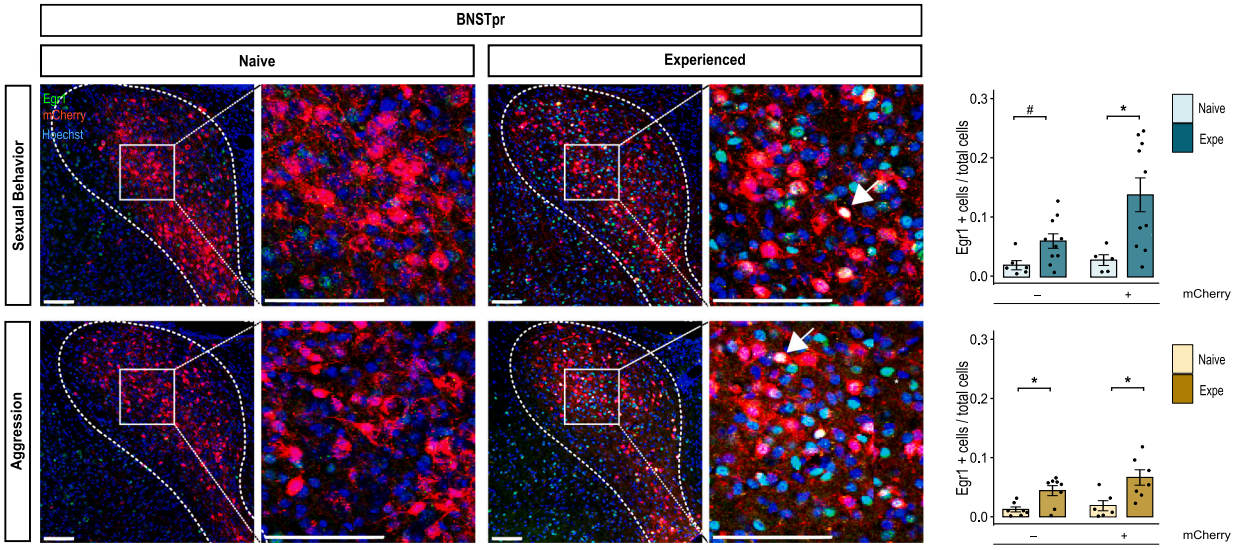

Fig. 9. Social experience triggers an activity increase in BNSTpr Aro + neurons projecting to the mPOA following territorial aggression and sexual behavior. Left, representative images of the retrograde tracing (mCherry, red) in the BNSTpr (dashed lines) co-stained with Egr1 (green) and Hoechst (blue). Arrows show double-labeled cells. Scale bars, 100 μM. Right, average proportion (per animal) of Egr1+ cells per slice that are mCherry positive (+) (mCherry signal >2sd) or negative (−) (mCherry signal <2sd) after sexual behavior (top) or territorial aggression (bottom). Error bars depicts SEM. All data from naive and experienced animals were compared using unilateral two-sample Fisher-Pitman permutation tests and Bonferroni corrected for 4 comparisons (2 cell populations x 2 brain regions); # $p = 0.08$, * $p \leq 0.05$.

mPOA, suggesting a crucial role of the MeApd as a substrate of neural plasticity following sexual behavior experience in male mice. Interestingly, Nordman et al. (2020) reported that strengthening synaptic activity in MeApv neurons that project to the BNSTpr or VMH, causes escalated aggression following training. These findings suggest that the MeA may also play an important role in enhancing aggression following experience via a sub-circuit implicating MeApv neurons projecting to the BNSTpr or the VMH. Our findings suggest that a specific plasticity in MeApd neurons, and notably those projecting to mPOA, may be more relevant for sexual experience than for aggression experience. Altogether, our data point to a differential sub-circuit plasticity after sexual vs aggression experience. Future studies aimed to specifically manipulate these sub-circuits after sexual or territorial aggression training will confirm the causal implication and the behavior-specificity of these effects.

We also observed altered sensory function in the VNO, which can be modulated by internal factors including hormonal changes and social experience, leading to behavioral changes in sexual behavior and pup care (Dey et al., 2015; Tachikawa et al., 2013). Sensory detection of social cues by the VNO is followed by MeA coding of the different olfactory stimuli to produce the appropriate behavioral response (Samuelsen and Meredith, 2009). We observe an increase in Egr1+ VSNs in experienced male mice following sexual behavior, but not after territorial aggression. This suggests that together with the MeApd and

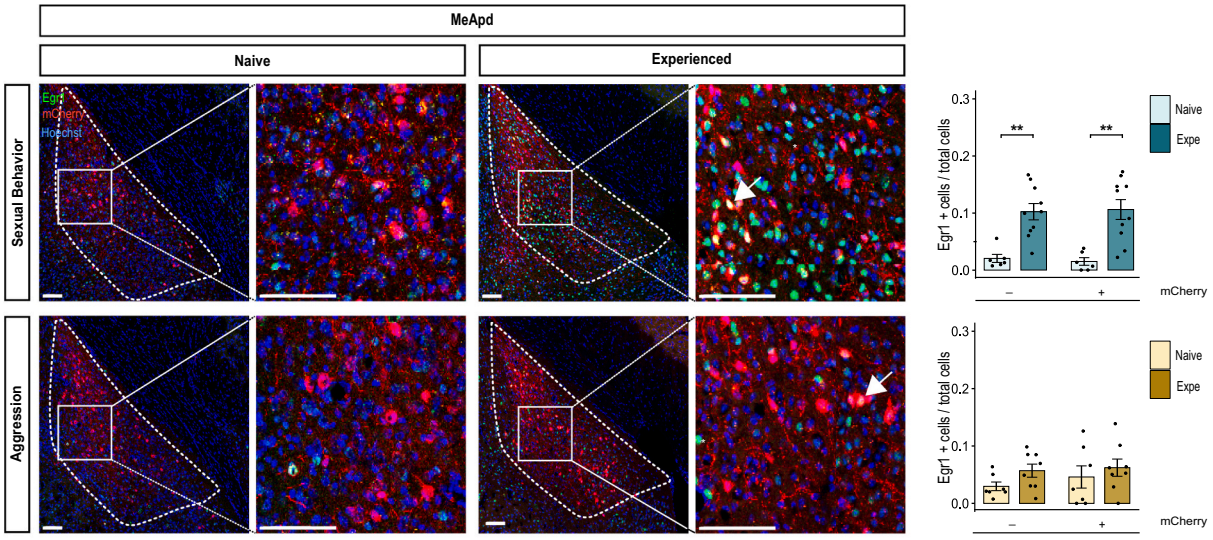

Fig. 10. Social experience triggers a behavior-specific activity increase in MeApd Aro + neurons projecting to the mPOA. Left, representative images of the retrograde tracing (mCherry, red) in the MeApd co-stained with Egr1 (green) and Hoechst (blue). Arrows show double-labeled cells. Scale bars, 100 μM. Right, average proportion (per animal) of Egr1+ cells per slice that are mCherry positive (+) (mCherry signal >2sd) or negative (−) (mCherry signal <2sd) after sexual behavior (top) or territorial aggression (bottom). Error bars depicts SEM. All data from naive and experienced animals were compared using unilateral two-sample Fisher-Pitman permutation tests and Bonferroni corrected for 4 comparisons (2 cell populations x 2 brain regions); ** $p \leq 0.01$.

mPOA, a specific sensory plasticity in the VNO may also play a role in sexual behavior learning.

Overall, our data indicate that in experienced animals, BNSTpr Aro + neurons are essential for the display of territorial aggression, but not for sexual behavior. In this context, we observe an increase in Egr1 activity in the MeApd, mPOA and VNO after sexual behavior, suggesting that social experience induces neural plasticity changes in these areas to modulate the behavior. One interesting question is whether the activation of these regions is only implicated in the display of sexual behavior in experienced animals or whether it is also required during the learning process. Our chemogenetic results suggest that the display of sexual behavior is less dependent on BNSTpr Aro + neurons in socially experienced animals, but we cannot exclude that the activation of BNSTpr Aro + neurons during sexual behavior in naive animals (Bayless et al., 2019) is required for sexual behavior learning. Future experiments aimed to inhibit MeApd Aro + cells vs BNSTpr Aro + cells in naive animals, during learning, will help to better understand the respective role of these regions in sexual behavior learning.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2025.105723.

CRediT authorship contribution statement

Elliott Trives: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Chantal Porte:** Writing – review & editing, Formal analysis, Data curation. **Thiago Seike Nakahara:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Matthieu Keller:** Writing – review & editing, Supervision, Conceptualization. **Hélène Vacher:** Writing – review & editing, Supervision, Conceptualization. **Pablo Chamero:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Ethics approval

All animal experiments have been approved by the local ethical committee (Comité d'Ethique en Expérimentation Animale Val de Loire) and complied with French and European guidelines (Apafis reference number 35299).

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Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

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Data availability

The datasets generated and analyzed during the current study, with detailed data analysis reports including used packages, codes and outputs are available in a G-Node repository (https://doi.gin.g-node.org/10.12751/g-node.cs3tdm/).

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