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Post-ejaculatory Inhibition of Female Sexual Drive via Heterogeneous Neuronal Ensembles in the Medial Preoptic Area

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eLife Assessment

This **important** work combines molecular genetics and behavioral analyses to identify inhibitory neurons in the female medial preoptic area as a neural locus that is activated following male ejaculation and whose prolonged activity plays a key role in the regulation of female sexual motivation. These experiments are rigorous and well-performed. The data are **compelling** and demonstrate that a subpopulation of neurons in the medial preoptic area are selectively activated following the completion of mating in females. The medial preoptic area has long been implicated as critical to sexual behavior in both sexes; however the use of a self-paced mating assay for females provides fine control over manipulating and monitoring cellular activity in this region during more naturalistic behavior. In addition, this study may act to inspire others to further explore the additional brain regions found to show upregulation of neural activity (Fos) during mating completion in females using the datasets generated here.

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Abstract

Summary

Male ejaculation acutely suppresses sexual motivation in male mice. In contrast, relatively little is known about how male ejaculation affects sexual motivation and sexual behavior in female mice. How the brain responds to completion of mating is also unclear. Here, by using self-paced mating assay, we first demonstrate that female mice show decreased sexual motivation acutely after experiencing male ejaculation. By using brain-wide analysis of activity-dependent labeling, we next pinpointed the medial preoptic area as a brain region strongly activated during the post-ejaculatory period. Furthermore, using freely moving *in*

vivo calcium imaging to compare neural activity of inhibitory and excitatory neurons in the medial preoptic area, we revealed that a subset of the neurons in this region respond significantly and specifically to male ejaculation but not to female-to-male sniffing or to male mounting. While there were excitatory and inhibitory neurons that showed increased response to male ejaculation, the response magnitude as well as the proportion of neurons responding to the event was significantly larger in the inhibitory neuron population. Next, by unbiased classification of their responses, we also found a subpopulation of neurons that increase their activity late after the onset of male ejaculation. These neurons were all inhibitory indicating that male ejaculation induces a prolonged inhibitory activity in the medial preoptic area. Lastly, we found that chemogenetic activation of medial preoptic area neurons that were active during post-ejaculatory period, but not during appetitive or consummatory periods, were sufficient to suppress female sexual motivation. Together, our data illuminate the importance of medial preoptic area as a brain node which encodes a negative signal that sustains low sexual motivation state after the female mice experience ejaculation.

Highlights

- Female mice show decreased sexual motivation in the post-ejaculatory period.
- A subset of MPOA neurons in female respond specifically to male ejaculation.
- Male-ejaculation evokes persistent activity in MPOA inhibitory neurons in females.
- Activation of a subset of MPOA neurons is sufficient to suppress female sexual motivation.

Introduction

Sexual behavior is a fundamental and universal component of the observable behavioral spectrum in all mammalian species. This behavior results in reproduction, the vital process for species propagation. However, reproduction carries significant burdens, particularly for females, spanning pregnancy, birth, nursing, offspring rearing, and heightened vulnerability to environmental threats. Consequently, the female ability to strictly control sexual drive is adaptive and confers individual survivability. This can involve both increasing and decreasing motivation to engage in sexual behavior, dependent on anticipated beneficial outcome. Rodents provide a powerful resource for studying female sexual behavior and the underlying neural basis (Agrati, 2022; Micevych and Meisel, 2017). Typically, rodent sexual behavior is divided into three periods: an appetitive, consummatory, and post-ejaculatory phase (Hashikawa et al., 2016; Ishii and Touhara, 2018; Lenschow and Lima, 2020). Importantly, in males, sexual motivation acutely reduces after ejaculation (Hull et al., 2005). However, less is known about how the experience of male ejaculation in female mice impacts their sexual behavior and motivation. Furthermore, how the transient experience of male ejaculation is represented in the brain to change the behavioral outcome is not well understood. The medial preoptic area (MPOA), a molecularly and functionally heterogenous brain region, has classically been known to regulate wide variety of social behaviors (Fang et al., 2018; Hashikawa et al., 2021; Karigo et al., 2021; Kohl et al., 2018; Moffitt et al., 2018; Tsuneoka and Funato, 2021; Wei et al., 2018). Indeed, the MPOA has also been described to be an important brain area for the regulation of female sexual behavior. Early studies using histological approaches to map neural activity in rats has also showed MPOA, together with other hypothalamic regions, to be activated during sexual behavior, after male ejaculation and after genital stimulations (Parada et al., 2010; Pfaus et al., 1996). Studies in female rats using electrophysiological recording or histological approaches

have reported that the MPOA contains neurons with various response specificity to sexual appetitive behaviors such as sniffing, consummatory behaviors such as male mounting, intromission, and ejaculation (Kato and Sakuma, 2000; Pfaus et al., 1993). Similarly, MPOA lesions increased sexual receptivity, suggesting that the MPOA negatively regulates female sexual behavior when intact (Powers and Valenstein, 1972). Interestingly, the MPOA has also been found to positively regulate female sexual appetitive behavior through their projection to the mesolimbic reward system (McHenry et al., 2017). However, how the MPOA both positively and negatively regulate female sexual behavior, furthermore, sexual motivation, has been a long-lasting question in the field. Here, we first assess sexual motivation in female mice by quantifying measurable approach or avoidance of a male conspecific. Next, by combining cellular-resolution whole-brain mapping of activity-dependent labeling, *in vivo* calcium imaging, and chemogenetic neural manipulation, we find that a subset of neurons in the MPOA, which are largely inhibitory, respond to male ejaculation but not to sexual appetitive behavior. This population is sufficient to suppress female sexual motivation. Our findings illuminate the importance of the MPOA as a brain region that encodes a negative signal that sustains low sexual motivation following male ejaculation in female mice.

Results

Female sexual motivation is suppressed after male ejaculation

Experimentally, sexual motivation can be quantified by the frequency of a subject displaying appetitive behavior, such as approach and sniffing behavior, toward an opposite sex conspecific. However, in a traditional rodent mating assay, where the female and male both freely interact with each other, it is difficult to isolate female-initiated appetitive behavior, and thus their sexual motivation. To directly address aspects of female sexual motivation independent from the male's behavior, we used a female self-paced mating assay (Erskine, 1989, 1985; Peirce and Nuttall, 1961). In this procedure, a female subject and a sexually experienced male are placed in a behavior apparatus divided by a wall with a hole small enough for a female to move through, but not the male. The female subject may move into the area where the male animal was placed ("Interaction zone") or move out to the other area ("Isolation zone") (**Figure 1A**). Thus, in this assay the female has direct control of when interaction happens and provides a more ethologically valid measure of female appetitive sexual behavior. Specifically, the amount of time the female subject spends in the interaction zone and the number of transitions they make serve as metrics to quantify female appetitive behavior. On the experiment day, the female subject, which was primed with estradiol to be behaviorally estrus, was placed in the apparatus with the partner ("Sexual interaction" trial) or alone ("Control" trial). Each animal went through both trials on different experiment days. The position of the animal was tracked to quantify the time spent in each zone. First, we found that the virgin female mice stayed in the isolation zone significantly longer than sexually experienced females, suggesting that previous sexual experience increased sexual motivation in the female mice (**Figure S1**). Next, in the sexually experienced animals, we observed robust behavioral changes after male ejaculation. First, we observed that female subjects tended to spend more time in the isolation zone after male ejaculation compared to post-sniffing, mounting and intromission (**Figure 1B–F**). To quantify the changes in animal behavior, we divided the sexual interaction trial into pre-and post-male ejaculation epochs (**Figure 1G–M**). As result, we found that there was a significant decrease in the number of zone transitions as well as a significant increase in the time spent in the isolation zone after male ejaculation. Importantly, this change was not dependent on the state of the male animal (**Figure S2**). When the female animal was introduced to a novel sexually motivated male animal immediately after they experienced male ejaculation from another mouse, they showed similar reduction in zone transition and increase in the time spent in the isolation zone. To further evaluate the female animal's motivation to engage in sexual behavior, we quantified the latency to return to the interaction zone after a behavioral episode (Bermant, 1961; Bermant and Westbrook, 1966).

This was the largest after the female experienced male ejaculation when compared to after sniffing or after mounting (**Figure 1I**). In addition, while there was no change in the amount of female-to-male sniffing, there was a significant decrease in the number of mounting and intromission episodes (**Figure 1J–L**). There was also a significant increase in the number of self-grooming behavioral episodes (**Figure 1M**). Animals that were in their natural estrus cycle showed similar behavior changes after experiencing male ejaculation (**Figure 1N–T**). Interestingly, animals that were in their natural estrus cycle showed strong reduction of sniffing after experiencing male ejaculation but no changes in self-grooming behaviors (**Figure 1Q**, **1T**). Collectively, these results suggest that male ejaculation acutely decreases sexual motivation.

Brain-wide mapping of neural response to male ejaculation using activity-dependent labeling in the female brain

We next questioned how male ejaculation suppresses female sexual motivation. To investigate this question, we first determined which brain regions were associated with neural activity during the post-ejaculatory period in an unbiased manner. We conducted a cellular-resolution brain wide activity mapping analysis using targeted recombination in active populations method (TRAP), which allows genetic labeling and access to neurons that are active during a specific time window determined by tamoxifen administration (DeNardo et al., 2019). To capture the activity pattern, Fos-2A-iCreER^{T2}::RCL-tdTomato (TRAP2::Ai14) female mice were administered 4-hydroxytamoxifen (4-OHT) immediately after male ejaculation ("post-ejaculatory" group) (**Figure 2A**). In this method, cells that show behaviorally induced Fos activity were labeled with tdTomato. In addition to these animals, we also prepared a group of animals that were administrated with 4-OHT while the animals were in the appetitive phase or in the consummatory phase. For the appetitive group, female subjects interacted with male mice and displayed female-to-male sniffing but did not experience any mount or intromission behavior, nor male ejaculation. For the consummatory group, female subjects interacted with male mice and displayed female-to-male sniffing, experienced mount or intromission behavior, but not male ejaculation. After 2 weeks, the tissue was collected, cleared, imaged, and processed for registration on a template brain atlas (Park et al., 2019; Renier et al., 2016) (**Figure 2A**). The tdTomato+ cells were segmented using a trained classifier and normalized by the area of each brain region (**Figure 2B**). As result, we found the level of tdTomato expression increased in a brain wide manner during the post-ejaculatory period when compared to the appetitive phase (**Figure 2C–D**, Table 2). We further noticed that regions well known to be related to social behavior had significantly higher tdTomato density in the post-ejaculatory group compared to appetitive group (**Figure 2E–I**, **Figure S3**, **S4**, Table 3). This includes paraventricular hypothalamic areas (PVHd, PVH, PaV) which are important for endocrinological regulations, medial amygdala (MeA) which is critical for olfactory sensory information processing, and the BNST (Flanigan and Kash, 2022; Marsh et al., 2021; Raam and Hong, 2021). The medial preoptic area nucleus (MPN), a subregion of the MPOA, had significantly higher tdTomato density in the post-ejaculatory group compared to the appetitive group. The MPN also had the 4th largest difference of density between the post-ejaculatory group and appetitive group among all the brain regions (**Figure 2F**). While it was not statistically significant, the consummatory group also tended to have a larger tdTomato density than the appetitive group in the MPN as well (**Figure 2G** and **2H**). From this result, we decided to further investigate the role of MPOA and how it is related to the regulation of female sexual motivation after experiencing male ejaculation.

Male ejaculation is represented by both inhibitory and excitatory cell types in the MPOA

MPOA is a molecularly heterogenous brain region. The area is a mixture of 80% inhibitory neurons and 20% of excitatory neurons which can be further classified into neuronal subtypes by marker gene expressions (Hashikawa et al., 2021; Moffitt et al., 2018). These subtypes are

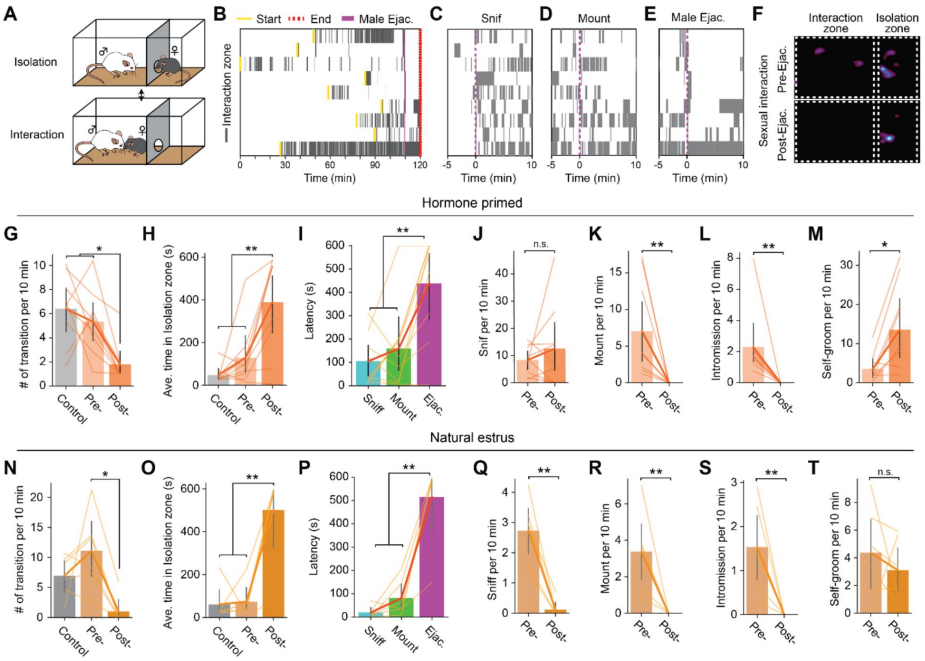


Figure 1

Female mice show decreased sexual motivation after male ejaculation.

(**A**) Schematic of female self-paced mating assay. The behavior apparatus was divided by a wall with a hole small enough for only the female subject to go through. The female subject was allowed to freely choose between "interaction" with the male partner in the larger zone or "isolation" in the smaller zone. Results using ovariectomized and hormone primed female mice are shown in **B – M**. Results using naturally estrus female mice are shown in **N – T**.
(**B**) Raster plots of time spent in the interaction zone (gray). The onset of male ejaculation, start and the endpoint of the experiment is shown in yellow, blue and red respectively.
(**C – E**) Raster plots of time spent in the interaction zone (gray) around the onset of the first female to male sniffing, male mounting, intromission and male ejaculation.
(**F**) Representative heatmap showing the time spent in the behavior apparatus during a control trial or a sexual interaction trial. The sexual interaction trial was further divided into pre-and post-male ejaculation.
(**G–H**) Behavior analysis for hormone primed animals. (**G**) Number of transitions between the zones per 10 minutes. RM ANOVA; F(2,16)=10.89, **p=0.001035889. Tukey's HSD; Control vs Post, **p=0.0015. Control vs Pre, p=0.6284. Post vs Pre, *p=0.0142. (**H**) Average time spent in the isolation zone during the control trial, and pre-and post-male ejaculation. RM ANOVA; F(2,16)=14.34, **p=0.00027. Control vs Post, ***p=0.0004. Control vs Pre, p=0.5361. Post vs Pre, **p=0.0057. (**I**) Latency to return to the interaction zone after sniff, male mount or male ejaculation. RM ANOVA; F(2,16)=11.87, **p=0.00069052. Ejaculation vs Sniff, **p=0.0016. Ejaculation vs Mount, **p=0.0077. Sniff vs Mount, p=0.7951. (**J**) Number of female-to-male sniffing per 10 minutes during pre-and post-male ejaculation. p=0.65234375. (**K**) Number of male mounting per 10 minutes during pre-and post-male ejaculation. **p=0.00390625. (**L**) Number of intromission per 10 minutes during pre-and post-male ejaculation. **p=0.00390625. (**M**) Number of female self-grooming per 10 minutes during pre-and post-male ejaculation. *p=0.025061844.
(**N–T**) Behavior analysis for naturally estrus animals. (**N**) Number of transitions between the zones per 10 minutes. RM ANOVA; F(2,10) = 7.54, *p=0.01007. Tukey's HSD; Control vs Post, p=0.0871. Control vs Pre, p=0.2727. Post vs Pre, **p=0.0038. (**O**) Average time spent in the isolation zone during the control trial, and pre-and post-male ejaculation. RM ANOVA; F(2,10) = 15.71, ***p=0.00082. Control vs Post, ***p=0.0002. Control vs Pre, p=0.9862. Post vs Pre, ***p = 0.0002. (**P**) Latency to return to the interaction zone after sniff, male mount, or male ejaculation. RM ANOVA; F(2,10) = 38.36, ***p=0.00002. Ejaculation vs Sniff, ***p=0. Ejaculation vs Mount, ***p=0. Sniff vs Mount, p=0.6201. (**Q**) Number of female-to-male sniffing per 10 minutes during pre-and post-male ejaculation. *p =0.03125. (**R**) Number of male mounting per 10 minutes during pre-and post-male ejaculation. *p=0.03125. (**S**) Number of intromission per 10 minutes during pre-and post-male ejaculation. *p=0.03125. (**T**) Number of female self-grooming per 10 minutes during pre-and post-male ejaculation. p=0.3452.
All data are shown as mean ± 95% confidence interval and were analyzed by RM ANOVA with Tukey HSD post-hoc test (FWER = 0.05) (**G–I** and **N–P**) or Wilcoxon signed-rank test (**J–M** and **Q–T**). n = 9 (**D–K**), n = 6 (**N–T**). *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

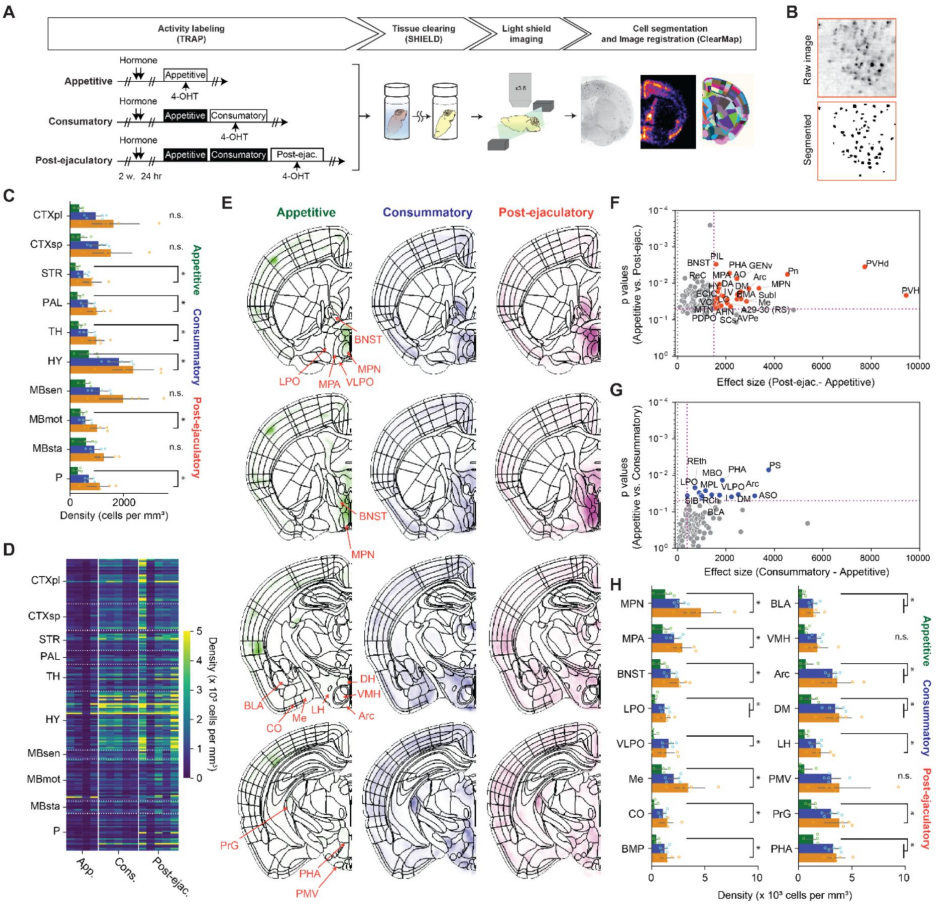


Figure 2

Brain wide analysis of cells responding to male ejaculation in the female brain.

(A) Overview of the experiment pipeline. After the activity labeling using TRAP, brain tissue was collected and cleared using SHIELD. The cleared tissue was imaged using a light-sheet microscope. The dataset was registered to a reference brain atlas and the number of tdTomato+ cells were quantified using ClearMap and ilastik.
(B) Representative image showing cell segmentation.
(C) Density of tdTomato+ cells per mm^3 in larger brain regions. CTXpl: Cortical plate, CTXsp: Cortical subplate, STR: Striatum, PAL: Pallidum, VERM: Vermal regions, HEM: Hemispheric regions, TH: Thalamus, HY: Hypothalamus, MBsen: Midbrain, sensory related, MBmot: Midbrain, motor related, MBsta: Midbrain, behavioral state related, P: Pons.
(D) Heatmap showing density of tdTomato+ cells per mm^3 in subregions. Each column indicates data from one subject. Each row indicates data from one subregion.
(E) Heatmap of average tdTomato density for each group from representative coronal planes.
(F) Scatterplot showing difference of average tdTomato density and p-value after Tukey's HSD post-hoc test between the post-ejaculatory group and the appetitive group.
(G) Scatterplot showing difference of average tdTomato density and p-value after Tukey's HSD post-hoc test between theconsummatory group and the appetitive group.
(H) Density of tdTomato+ cells per mm^3 in a subset of subregions. The full list of brain regions is shown in **Figure S2**.
MPN: Medial preoptic nucleus, MPA: Medial preoptic area, BNST: Bed nuclei of the stria terminalis, LPO: Lateral preoptic area, VLPO: Ventrolateral preoptic nucleus, Me: Medial amygdaloid nucleus, CO: cortical amygdaloid nucleus, BMP: Basomedial amygdaloid nucleus, posterior part, BLA: Basolateral amygdaloid nucleus, anterior part, BLP: Basolateral amygdaloid nucleus, posterior part, VMH: Ventromedial hypothalamic nucleus, Arc: Arcuate hypothalamic nucleus, DM: Dorsomedial hypothalamic nucleus, LH: Lateral hypothalamic area, PMV: Premamillary nucleus, ventral part, PrG: Pregeniculate nucleus of the prethalamus, PHA: Posterior hypothalamic area.
All data are shown as mean ± 95% confidence interval and were analyzed by ANOVA test with Tukey's HSD post-hoc test (FWER = 0.05) (**C**: FDR = 0.05, **I**: FDR = 0.10). Detailed statistical values are shown in Table 2 and Table 3. Appetitive (green), n = 4. Consummatory (blue), n = 5. Post-ejaculatory (orange), n = 5. *$p < 0.05$, ns, not significant.

often associated with a biological function. One example are the *Nts* expressing cells, which also tend to co-express *Esr1* and *Vgat* (McHenry et al., 2017). In female mice, these neurons were shown to respond to male odor when the subject was sexually receptive, and the activation of these neurons were sufficient to increase sexual appetitive behaviors. Therefore, identifying the specific cell types which respond to male ejaculation but not to appetitive behavior is critical to understand how the MPOA complex regulates female sexual motivation. Here, we utilized multiplexed *in situ* RNA hybridization chain reaction (HCR) to identify previously characterized cell type markers of MPOA and an immediate early gene *Fos* (**Figure S5A**) (Choi et al., 2014). Brain samples were collected after the subject experienced male ejaculation ("post-ejaculatory" group) or during the appetitive phase, while the subject was interacting with the male mice without any consummatory behavior ("Appetitive" group) (**Figure S5B**). First, to understand which subregion of the preoptic area was activated, we quantified the number of *Fos*+ cells. As result, we found that the number of *Fos*+ cells in the post-ejaculatory group were significantly higher than appetitive group in the MPN but not in the nearby regions (**Figure S5C–D**). In the MPN, the amount of *Vglut2*+ and *Vgat*+ cells expressing *Fos* were significantly higher in the post-ejaculatory group than in the appetitive group, suggesting that male ejaculation activates both excitatory and inhibitory neurons (**Figure S5E–I**). The proportion of *Fos*+ cells was higher in *Vgat*+ population. To further investigate the cell types in the MPN that were activated by male ejaculation, we analyzed the co-expression of *Fos* and the MPOA cell type markers (**Figure S5J**). As result, in the *Vglut2*+ expressing cells, we found that *Fos* induction was only observed in the cells that co-expressed *Calcr*. In contrast, in the *Vgat*+ expressing cells, *Fos* induction was observed in *Calcr*, *Gal* and *Prlr* co-expressing cells. Next, we conducted clustering on the gene expression matrix to classify cells and analyzed the distribution of *Fos* among the clusters (**Figure S5K–M**). As result, we were able to identify 6 potential cell types which we labeled as type 1: *Vglut2*+, type 2: *Vgat*+, type 3: *Gal*+, type 4: *Nts*+, type 5: *Calcr*+ and type 6: NA, or a cell group that could not be categorized by a specific gene expression pattern. Type 1: *Vglut2*+, type 2: *Vgat*+ and type 3: *Gal*+ had significantly more *Fos*+ cells from the post-ejaculatory group compared to the appetitive group (**Figure S5M**). In contrast, *Fos*+ cells were not induced in type 4: *Nts*+ indicating that MPOA neurons which regulate appetitive sexual behavior was not activated by male ejaculation. Collectively, these results indicate that male ejaculation induces more neural activity in the MPN when compared to appetitive behavior, both in excitatory and inhibitory cell types.

A subpopulation of MPOA Vgat cells respond to male ejaculation in the female brain

While mapping neural activity based on *Fos* activity provided critical insight in how the brain responds to male ejaculation, the low temporal resolution of the method makes it difficult to identify cells that specifically respond to the onset to each behavior. For this reason, we next conducted single-cell resolution *in vivo* imaging in a freely moving animal (**Figure 3A–B**). We virally labeled MPOA inhibitory and excitatory cells with a calcium indicator GCaMP6s by using a Cre-dependent virus and female Vgat-Cre or Vglut2-Cre respectively (**Figure 3C**, **Figure S6**) (Vgat-Cre: n = 6, 87 ± 27 cells. Vglut2-Cre: n = 4, 68 ± 42 cells). We recorded the calcium activity of MPOA Vgat and Vglut2 cells in female mice during mating in a home cage mating assay (**Figure 3E**). First, we analyzed the magnitude of calcium activity for 15-seconds after behavior onset. From peri-event analysis, we found that as a population, Vgat cells showed significant changes in calcium activity after the onset of all behaviors observed, with the highest increase after male ejaculation (**Figure 3F–G**). Vglut2 cells showed increased activity after sniffing and mounting, intromission, and male ejaculation. Furthermore, when we compared the response of Vgat and Vglut2 cells, Vgat cells showed significantly higher average response magnitude after sniffing, self-grooming, anogenital sniffing and male ejaculation than the Vglut2 cells (**Figure 3H**). Vgat cells also contained a significantly higher proportion of cells that positively respond (> 28) to sniff and male ejaculation onset (**Figure 3I**). These results suggest that male ejaculation signal is more dominantly represented in the MPOA inhibitory cells than in the excitatory cells. On the other hand, the response magnitude and the proportion of cells that respond after mounting and

intromission had no significant difference between the two cell types. This indicates that the excitatory/inhibitory balance in the MPOA fluctuates during mating. The MPOA has high inhibitory activity during appetitive phase, with more Vgat cells responding to sniffing, similar activity during the consummatory phase, and higher inhibitory activity after male ejaculation.

To further investigate the heterogeneity of MPOA during mating, we subset the cells into those which positively responded to any of sniff, mount or male ejaculation, and then classified the cells based on their specificity (**Figure 4A,B** and **Figure S7**). Consistent with **Figure 3I**, the number of cells that specifically responded was largest to male ejaculation with 177 cells, next to mount with 46 cells and to sniff with 12 cells. When compared to other cell populations, sniff cells and completion cells only showed significantly stronger responses than the other cells to sniff and male ejaculation respectively (**Figure 4C**). Mount responsive cells showed significantly stronger response than the other cells to mount, intromission and anogenital sniffing, but not to sniffing and male ejaculation (**Figure 4C**). We also found that the cells that respond to these 3 behaviors partially overlap but were mostly distinct (**Figure 4D**). The proportion of cells that respond specifically to a behavior was highest for male ejaculation (Vgat: 80%, Vglut2: 82%), and lower in mounting (Vgat: 55%, Vglut2: 68%) and sniffing (Vgat: 36%, Vglut2: 0%). There was no notable bias in the anatomical distribution of these cells within the field of view (**Figure 4E**). Overall, these results suggest that the MPOA contains highly heterogenous functional population which contains a large proportion of cells specifically responding to male ejaculation in female mice.

MPOA Vgat cells display prolonged activity after the onset of male ejaculation

Female mice avoid sexual interaction with a male animal after male ejaculation for hours and even days, suggesting a persistent changes in the animal's behavior (McGill and Coughlin, 1970; Zhou et al., 2023). How the MPOA regulate this persistent change in female sexual motivation is unclear. To analyze the response of MPOA cells on a longer time scale, we focused on the cells that positively responded to male ejaculation and characterized the calcium response pattern for 120-seconds after the onset (**Figure 5**). First, we observed that Vgat cells showed more diverse response patterns during this time-window (**Figure 5A,B**). To quantify this difference, we calculated the decay length of calcium signal, the time for the increased calcium signal to return to baseline (**Figure 5C**). As result, we found that Vgat cells had significantly longer decay length compared to the Vglut2 cells, suggesting increased activity late after the onset of male ejaculation. To further classify the temporal dynamics of responses to male ejaculation, we first concatenated calcium activity that occurred within this time-window from both Vgat and Vglut2 cells and conducted spectral clustering in an unbiased fashion. As a result, we were able to classify the responses into 5 types that could be characterized as: 1) fast response, 2) slow response, 3) late response, 4) weak response and 5) negative response (**Figure 5E**, **Figure S8**). Type 3 contained cells that show increased calcium dynamics late after the onset of male ejaculation and had significantly larger decay length than the other cell types (**Figure 5F**). Then we separated the Vgat and Vglut2 cells to compare their proportions within each cluster. Interestingly, all the cells in type 3 were Vgat cells, suggesting that the increased activity in the MPOA late after the onset of male ejaculation is mediated by inhibitory neurons (**Figure 5H–K**). In contrast, Vglut2 cells were more likely to show fast responses than Vgat cells (**Figure 5K**). Collectively, these results suggest that the MPOA contains a subpopulation of inhibitory neurons that increase their activity late after the onset of male ejaculation, which may contribute to persistent suppression of the female animals' sexual motivation.

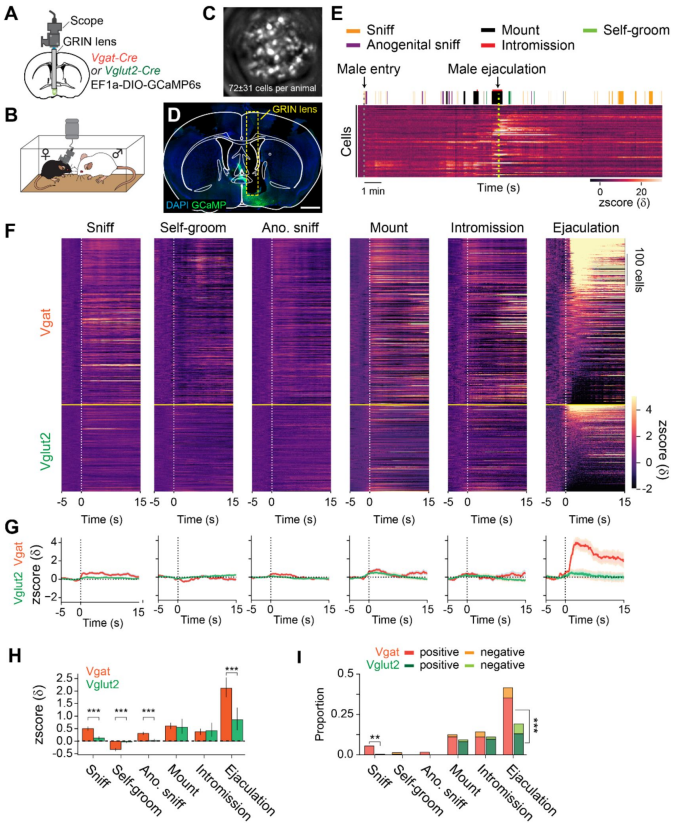


Figure 3

Male ejaculation signal is strongly represented in the MPOA of the female mice.

(A) Schematic of *in vivo* calcium imaging from MPOA Vgat and Vglut2 neurons. AAVDj EF1a-DIO-GCaMP6s was injected into the MPOA of Vgat-Cre or Vglut2-Cre female mice. A GRIN lens was placed above the MPOA following virus injection.
(B) Calcium activity was imaged from a female subject during a free moving mating assay with a sexually experienced male partner.
(C) Representative maximum projected image of the field of view during calcium imaging. Vgat: 87 ± 28 cells, n = 6. Vglut2: 68 ± 42 cells, n = 4.
(D) Representative coronal section showing GCaMP6s expression and GRIN lens placement.
(E) Representative raster plot showing behavior events during the mating assay and a heatmap showing calcium activity from cells imaged during the assay. Behaviors quantified: female to male sniffing (Sniff), female self-grooming (Self-groom), male to female anogenital sniffing (Ano. sniff), male mounting (Mount), male intromission (Intromission) and male ejaculation (Ejaculation).
(F) Heatmap showing peri-event calcium activity around behavior onset. Cells are ordered by the average response after male ejaculation.
(G) Line plot showing average calcium activity around behavior onset. Vgat neurons, blue. Vglut cells, orange.
(H) Average calcium response magnitude following behavior onset per cell. Vgat-Cre: n = 522 cells, Vglut2-Cre: n = 271 cells. Sniff: t(791)=6.46, ***p=1.08E-09. Grooming: t(791)=-7.25, ***p=5.89E-12. Male anogenital sniffing: t(791)=6.64, ***p=3.41E-10. Mount: t(791)=0.34, p=1. Intromission: t(791)=-0.32, p=1. Ejaculation: t(791)=4.00, ***p=0.00042.
(I) Proportion of cells which responded positively or negatively to behavior onset (>28). Positive: Sniff; Vgat 29/522 cells vs. Vglut2 1/271 cells, **p=0.00169. Grooming; Vgat 2/522 cells vs. Vglut2 0/271 cells, p=1. Male Anogenital Sniffing; Vgat 9/522 cells vs. Vglut2 0/271 cells, p=0.178. Mount; Vgat 58/522 cells vs. Vglut2 22/271 cells, p=1. Intromission; Vgat 57/522 cells vs. Vglut2 26/271 cells, p=1. Ejaculation; Vgat 184/522 cells vs. Vglut2 35/271 cells, ***p=1.52E-10. Negative: Sniff; Vgat 0/522 cells vs. Vglut2 0/271 cells, no statistic. Grooming; Vgat 5/522 cells vs. Vglut2 0/271 cells, p=0.636. Male Anogenital Sniffing; Vgat 0/522 cells vs. Vglut2 0/271 cells, no statistic. Mount; Vgat 7/522 cells vs. Vglut2 3/271 cells, p=1. Intromission; Vgat 17/522 cells vs. Vglut2 4/271 cells, p=0.831. Ejaculation; Vgat 33/522 cells vs. Vglut2 17/271 cells, p=1.
All data are shown as mean ± 95% confidence interval and were analyzed by Student's t-test with Bonferroni correction (**H**) and chisquare test with Bonferroni correction (**I**). *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

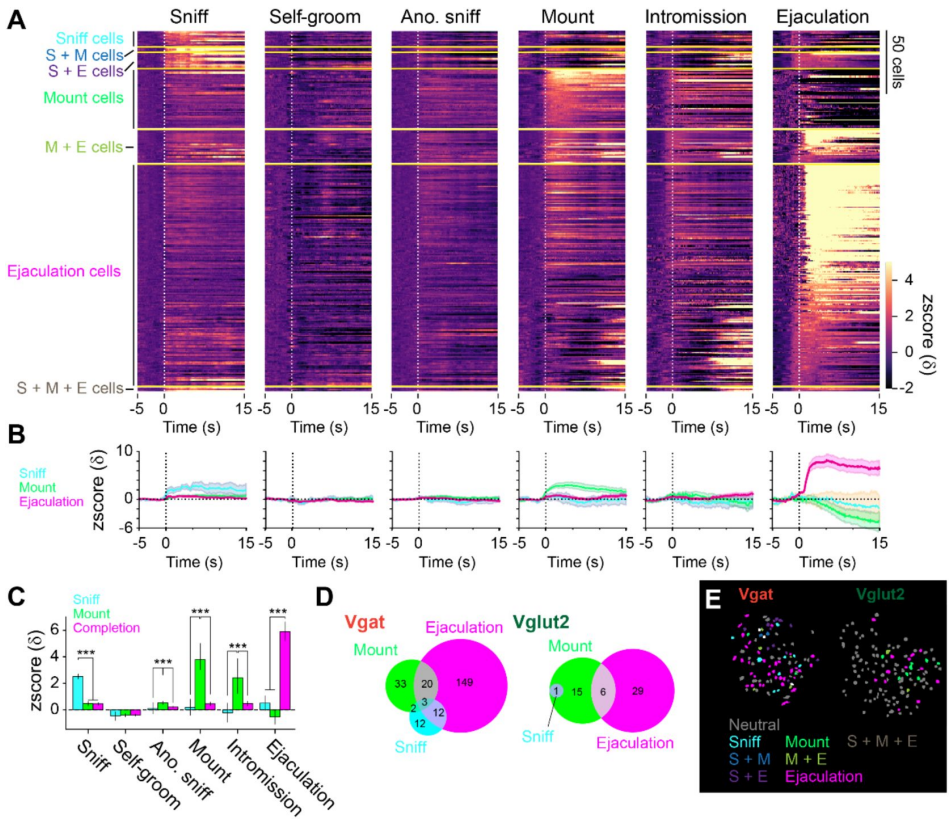


Figure 4

Heterogenous response properties to sexual behaviors in the MPOA.

(A) Heatmap showing peri-event calcium activity around behavior onset of cells that were categorized based on response magnitude. S: Sniff, M: Mount, E: Ejaculation.
(B) Line plot showing average calcium activity around behavior onset. Sniff cells, blue. Mount cells, green. Ejaculation cells, magenta.
(C) Average calcium response magnitude following behavior onset per cell in each cell types. Sniff: One-way ANOVA, $F(2,235)=73.35$, ***p=1.77E-25. Tukey's HSD test, Ejaculation cells vs Mount cells, p=0.9996. Ejaculation cells vs Sniff cells, ***p=0. Mount cells vs Sniff cells, ***p=0. Grooming: One-way ANOVA, $F(2,235)=0.09$, p=0.915. Tukey's HSD test, Ejaculation cells vs Mount cells, p=0.9723. Ejaculation cells vs Sniff cells, p=0.9236. Mount cells vs Sniff cells, p=0.9695. Male Anogenital Sniffing: One-way ANOVA, $F(2,235)=12.34$, ***p=8.01E-06. Tukey's HSD test, Ejaculation cells vs Mount cells, ***p=0. Ejaculation cells vs Sniff cells, p=0.6794. Mount cells vs Sniff cells, **p=0.0048. Mount: One-way ANOVA, $F(2,235)=68.32$, ***p=4.1E-24. Tukey's HSD test, Ejaculation cells vs Mount cells, ***p=0. Ejaculation cells vs Sniff cells, p=0.8726. Mount cells vs Sniff cells, ***p=0. Intromission: One-way ANOVA, $F(2,235)=15.72$, ***p=3.9E-07. Tukey's HSD test, Ejaculation cells vs Mount cells, ***p=0. Ejaculation cells vs Sniff cells, p=0.5451. Mount cells vs Sniff cells, ***p=0.0009. Ejaculation: One-way ANOVA, $F(2,235)=49.59$, ***p=1.08E-18. Tukey's HSD test, Ejaculation cells vs Mount cells, ***p=0. Ejaculation cells vs Sniff cells, ***p=0.0001. Mount cells vs Sniff cells, p=0.7295.
(D) Venn diagram showing the number of cells with positive and negative response in each cell type. (>28).
(E) Representative field of view showing spatial location of cells with different response properties to sexual behavior in Vgat (left) and Vglut2 (right) cells.
All data are shown as mean ± 95% confidence interval and were analyzed by Student's t-test with Bonferroni correction (**C**). Sniff cells: n = 12 cells. Mount cells: n = 48 cells. Ejaculation cells: n = 178 cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $***p < 0.001$; ns, not significant.

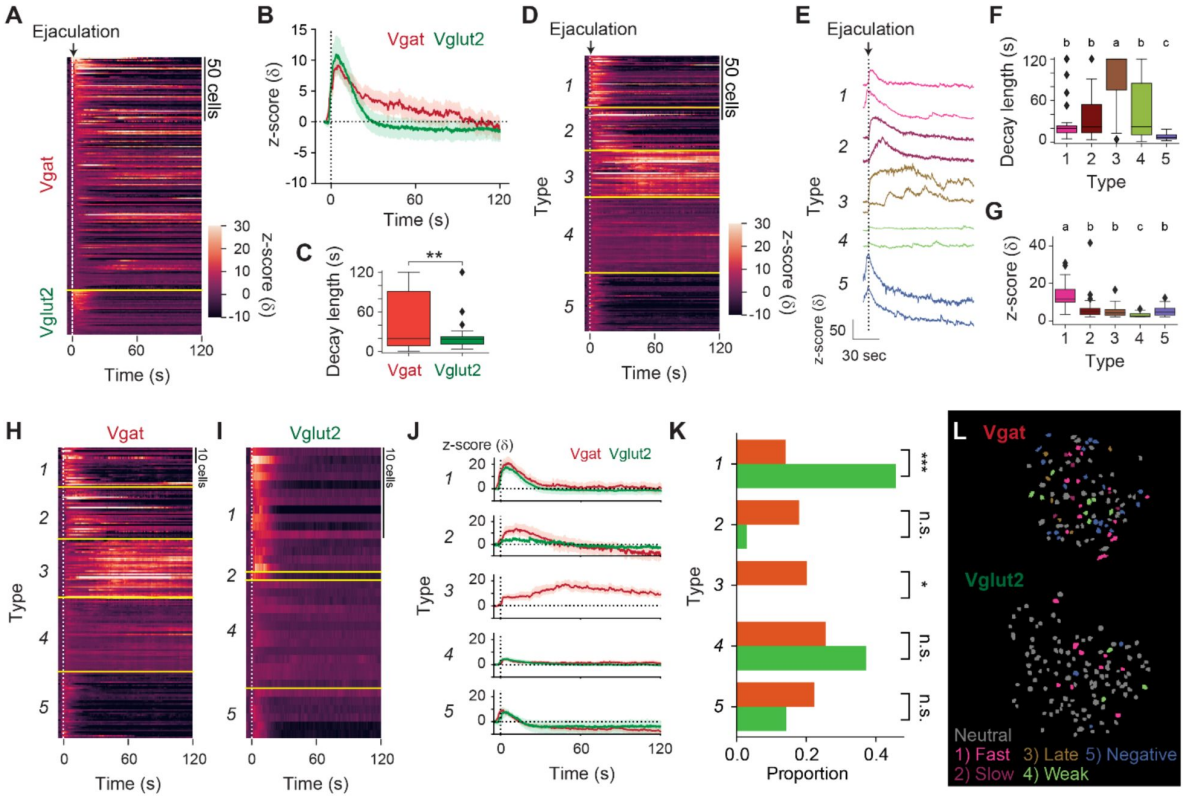


Figure 5

MPOA inhibitory neurons display prolonged activity late after the onset of male ejaculation in female mice.

(A) Heatmap showing peri-event calcium activity around male ejaculation onset for male ejaculation-responding cells in a longer time scale.
(B) Line plot showing average calcium activity of Vgat and Vglut2 population around behavior onset.
(C) The decay length for male ejaculation-responding cells from Vgat and Vglut2 population. Vgat-Cre: n = 184 cells. Vglut2-Cre: n = 35 cells. t(217)=3.039653717, **p=0.002659548.
(**D–G**) Cluster based analysis of the activity pattern of the male ejaculation-cells.
(D) Heatmap showing peri-event calcium activity around male ejaculation onset sorted by cluster.
(E) Representative calcium activity traces of cells from each cluster.
(F) The decay length for male ejaculation-responding cells from each cluster. Further details of statistical tests are shown in Table 1.
(G) Average z-scored response for male ejaculation-responding cells from each cluster. Further details of statistical tests are shown in Table 1.
(**H–K**) Cluster based analysis of the activity pattern of the male ejaculation-cells in Vgat and Vglut2 population.
(**H–I**) Heatmap showing peri-event calcium activity of Vgat (H) and Vglut2 (I) cells around male ejaculation onset sorted by cluster.
(J) Line plot showing average calcium activity of Vgat and Vglut2 cells in each cluster around behavior onset.
(K) Proportion of Vgat and Vglut2 cells in each cluster over Vgat and Vglut2 cells in all clusters. Statistical results are shown next to the cluster name. Type1; Vgat 26/184 cells vs Vglut2 16/35 cells, ***p=6.80E-05. Type2; Vgat 33/184 cells vs Vglut2 1/35 cells, p=0.119810823. Type3; Vgat 37/184 cells vs Vglut2 0/35 cells, *p=0.018. Type4; Vgat 47/184 cells vs Vglut2 13/35 cells, p=0.792188347. Type5; Vgat 41/184 cells vs Vglut2 5/35 cells, p=1.
(L) Representative field of view showing spatial location of cells with different response properties after male ejaculation in Vgat (left) and Vglut2 (right) cells All data are shown as mean ± 95% confidence interval and were analyzed by Student's t-test (**C**), ANOVA with post-hoc Tukey HSD test (**F** and **G**), and chisquare test with Bonferroni correction (**K**). Type 1: n = 42 cells, Vgat-Cre: n = 26 cells, Vglut2: n = 16 cells. Type 2: n = 34 cells, Vgat-Cre: n = 33 cells, Vglut2: n = 1 cells. Type 3: n = 37 cells, Vgat-Cre: n = 37 cells, Vglut2: n = 0 cells. Type 4: n = 60 cells, Vgat-Cre: n = 47 cells, Vglut2: n = 13 cells. Type 5: n = 46 cells, Vgat-Cre: n = 41 cells, Vglut2: n = 5 cells.
(**C** and **K**) *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant. (**F** and **G**) a vs. b: p < 0.001. b vs. c: p < 0.05. a vs. c: p < 0.001.

An activity defined subset of neurons in the MPOA is sufficient to suppress female sexual behavior

Next, we examined whether the increased activity of neurons in the MPOA is sufficient to suppress female sexual motivation after male ejaculation. We injected an AAV to express the chemogenetic neural activator hM3Dq or eYFP as control into the MPOA of TRAP2 female mice (**Figure 6A**). To capture the neural activity, each mouse was administrated 4-OHT immediately after the after male ejaculation ("post-ejaculatory-hM3Dq" or "post-ejaculatory-eYFP" group). In addition to these animals, we also prepared a group of animals in which we injected an AAV expressing hM3Dq and then administrated 4-OHT during the appetitive phase ("appetitive-hM3Dq" group) or the consummatory phase ("consummatory-hM3Dq" group) (**Figure 6C**). We found that while the overall number of TRAP-labeled cells had no difference between the post-ejaculatory groups, consummatory group and appetitive group, the proportion of cells located in the MPN was significantly higher in the post-ejaculatory group (**Figure 6D** and **Figure S9A–C**). From *in situ* RNA hybridization, we found that most TRAP-labeled cells in the MPN were *Vgat*+ (*Vgat*+: 0.76 ± 0.16. *Vglut2*+: 0.15 ± 0.13.). The proportion of *Vgat*+ cells was significantly higher than the *Vglut2*+ cells (**Figure S7D–G**). Together these results suggest that the post-ejaculatory group labels more inhibitory neurons in the MPN than in appetitive group. Furthermore, we analyzed the accuracy and efficiency of TRAP labeling by allowing the female subject to experience male ejaculation before perfusion (**Figure S9H–L**). By immunolabeling c-Fos protein, we analyzed how well the TRAP ensemble overlaps with the c-Fos positive-ensemble in the MPN. As a result, we first found that the proportion of c-Fos+ and TRAP-labeled cells in the post-ejaculatory group tended to be higher than the appetitive group and the consummatory group (**Figure S9I**). The proportion of c-Fos+ cells were consistent across each group (**Figure S9J**). Next, we found that the accuracy of TRAP labeling (the proportion of TRAP-labeled cells that were also c-Fos positive) was similar between the groups (**Figure S9K**). The efficiency of TRAP labeling (the proportion of c-Fos+ cells that were also TRAP labeled) tended to be higher in the post-ejaculatory group (**Figure S9L**) than in the appetitive group and the consummatory group. This also suggests that the appetitive group labels a different set of neurons than the post-ejaculatory group. These results indicate that by using the TRAP2 method, we were able to specifically label a proportion of MPOA neurons, which are primarily inhibitory, that contains neurons that respond to male ejaculation.

To test the causality of these neurons, we first investigated how the activation affects female sexual behavior in a home cage mating assay (**Figure S10A–F**). The subjects were intraperitoneal injection (i.p.) injected with CNO 30-minutes before the assay. As result, first we found that post-ejaculatory-hM3Dq group but not the appetitive-hM3Dq and consummatory-hM3Dq group showed significantly fewer sniffing events compared to post-ejaculatory-eYFP group (**Figure S10C**). For consummatory behaviors, while post-ejaculatory-hM3Dq group showed no difference in mounting episodes compared to post-ejaculatory-eYFP group, there were significantly less intromission episodes, resulting in lower receptive scores (**Figure S10C–F**). Thus, from these results, activation of MPOA neurons that respond to male ejaculation suppresses both appetitive and consummatory sexual behavior in female mice. As a next step, we investigated how the activation of these MPOA neurons affects female sexual motivation in the self-paced mating assay (**Figure 6**). The subjects were i.p. injected with CNO or saline 30-minutes before the assay (**Figure 6B**). As result, only post-ejaculatory-hM3Dq group showed significantly fewer number of transitions and spent more time in the isolation zone on the CNO trial than on the saline trial (**Figure 6E–G**). We also found that post-ejaculatory-hM3Dq group displayed fewer numbers of self-grooming, mounting, intromission and lower receptivity score on the CNO trial than on the saline trial (**Figure 6H–L**). Interestingly, activation of these neurons did not induce conditional place preference or aversion (**Figure S10G–J**), implying that the suppression of sexual behavior is not associated with negative valence. Next, we investigated how inhibition of these neurons affects female sexual behavior (**Figure S11**). We prepared a new group of mice in

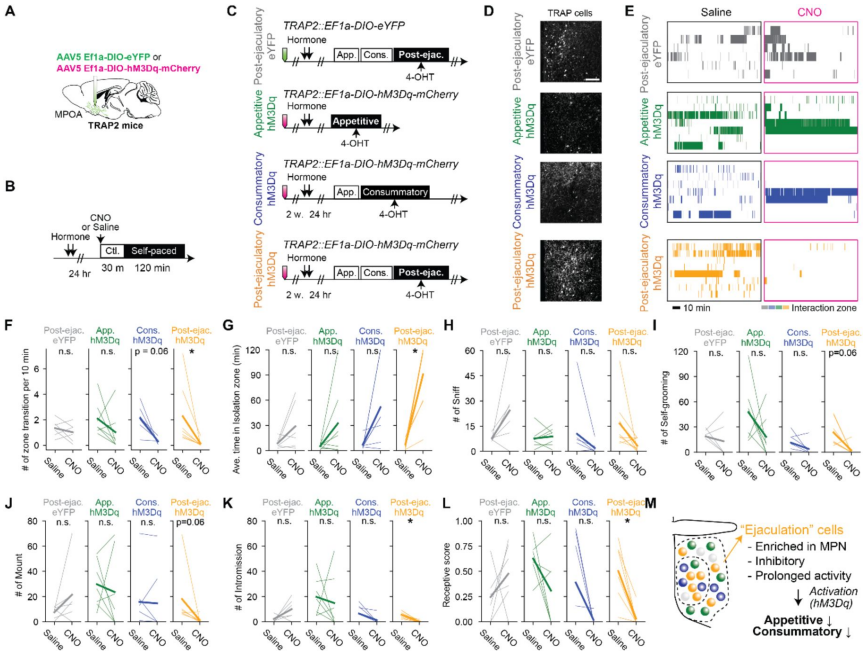


Figure 6

Activation of a subpopulation of MPOA labeled following mating is sufficient to suppress female sexual behavior.

(A) Schematic of selective labeling of MPOA neurons using TRAP2. AAV5 EF1a-DIO-hM3Dq-mCherry or AAV5 EF1a-DIO-eYFP as control was injected into the MPOA of the female TRAP2 subjects. After incubation of the virus, the female subjects went through procedures to TRAP cells in MPOA (further described in **C**).
(B) Schematic of pharmaco-genetic activation of MPOA neurons during a female self-paced mating assay. Female subjects were primed with hormone before the experiment day. On the experiment day, subjects were injected i.p. with CNO to activate TRAP cells, or saline as control. 30 minutes after the injection, the male partner was placed into the apparatus and the animal behavior was recorded for 2 hrs.
(C) After incubation of the virus, the female subjects were administered with hormones, then received an ejaculation from the male partner or only interacted without mating. Immediately after, the subjects were administrated with 4-hydroxytamoxifen (4-OHT).
(D) Representative image showing TRAP cells MPN from post-ejaculatory-eYFP, appetitive-hM3Dq and post-ejaculatory-hM3Dq group. Further histological analysis is shown in **Figure S9**. Scale bar, 100 μm.
(E) Raster plots of time spent in the interaction zone the female self-paced mating assay (post-ejaculatory-eYFP: gray. appetitive-hM3Dq: green, consummatory-hM3Dq: blue, post-ejaculatory-hM3Dq: orange).
(F) Number of zone transitions per 10 minutes. Post-ejaculatory-hM3Dq; *p=0.03125. Post-ejaculatory-eYFP; p=1. Appetitive-hM3Dq; p=0.875. Consummatory-hM3Dq; p=0.0625.
(G) Average time in isolation zone. Post-ejaculatory-hM3Dq; *p=0.03125. Post-ejaculatory-eYFP; p=1. Appetitive-hM3Dq; p=0.875. Consummatory-hM3Dq; p=0.125.
(H) Number of female-to-male sniff. Post-ejaculatory-hM3Dq; p=0.11197. Post-ejaculatory-eYFP; p=0.375. Appetitive-hM3Dq; p=1. Consummatory-hM3Dq; p=0.4594.
(I) Number of female self-grooming. Post-ejaculatory-hM3Dq; p=0.0625. Post-ejaculatory-eYFP; p=1. Appetitive-hM3Dq; p=1. Consummatory-hM3Dq; p=0.552.
(J) Number of male mount. Post-ejaculatory-hM3Dq; p=0.0625. Post-ejaculatory-eYFP; p=0.875. Appetitive-hM3Dq; p=1. Consummatory-hM3Dq; p=1.
(K) Number of male intromission. Post-ejaculatory-hM3Dq; *p=0.03125. Post-ejaculatory-eYFP; p=0.625. Appetitive-hM3Dq; p=1. Consummatory-hM3Dq; p=0.2716.
(L) Receptive score (number of mount episodes with intromissions/number of all mount episodes). Post-ejaculatory-hM3Dq; p=0.03125. Post-ejaculatory-eYFP; p=1. Appetitive-hM3Dq; p=0.625. Consummatory-hM3Dq; p=0.2716. (**M**) Schematic illustration summarizing how MPOA neurons suppress female sexual motivation after male ejaculation.
All data are shown by thin lines for individual subjects and thick lines for mean. The data were analyzed by Wilcoxon rank sum test with Bonferroni correction (**F–L**). post-ejaculatory-eYFP: n = 6, appetitive-hM3Dq: n = 7, consummatory-hM3Dq: n = 8. post-ejaculatory-hM3Dq: n = 8. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

which we injected an AAV expressing hM4Di or eYFP as control in TRAP2 female mice. Two-weeks after injection, we administrated 4-OHT immediately after the subject experienced male ejaculation ("post-ejaculatory-hM4Di" or "post-ejaculatory-eYFP" group). First, we tested the animals in the self-paced mating assay (**Figure S11A–L**). The female subjects were i.p. injected with CNO or saline 30-minutes before the assay (**Figure S11B**). As result, the subjects did not show any significant change in sexual behavior during this assay (**Figure S11D–L**). Next, we tested the animals in the home cage mating assay (**Figure S11M–S**). The subjects were intraperitoneal injections (i.p.) injected with CNO 30-minutes before the assay (**Figure S11M**). As result, the post-ejaculatory-hM4Di group experienced did not show significant changes in sniffing, self-grooming or mounting, but showed more intromission and higher sexual receptivity than in the post-ejaculatory-eYFP group (**Figure S11O–S**). Collectively, these results suggests that the MPOA neurons are sufficient but not necessary for the modulation for sexual motivation but is sufficient and necessary for consummatory behaviors in female mice (**Figure 6M**).

Discussion

How sexual motivation is represented in the brain has been a long-lasting question in the field. Here we found that MPOA plays a critical role in regulating female sexual motivation. We found that MPOA neurons, especially the inhibitory neurons, respond significantly and specifically to male ejaculation. These neurons show persistent activity after the onset of male ejaculation. Reactivation of these neurons result in suppression of female sexual motivation. We propose that MPOA together with other neural substrates such as the BNST (Zhou et al., 2023), encode a negative feedback signal that sustains low sexual motivation state after male ejaculation in female mice. Here, we will further discuss how sensory inputs contribute to the male ejaculation response in the MPOA, how MPOA responds to different sexual behaviors and how these neurons work in concert with other sexual behavior related hypothalamic regions.

Using self-paced mating paradigm to evaluate sexual motivation in female rodents

The self-paced mating paradigm is a classical yet powerful method to directly assess female sexual behaviors (Erskine, 1989, 1985; Peirce and Nuttall, 1961). This paradigm is still in current use, mainly in rats (Ventura-Aquino and Paredes, 2023). Our paper demonstrate that this simple behavioral paradigm is still a powerful method to study neural circuitry of female sexual behavior and motivation in mice. In our results we first demonstrate that the latency to return to the interaction zone was significantly larger after the animal experienced male ejaculation consistent with the results shown in rats. Direct measurements of both increase of withdrawal and decrease of approach to sexual behavior are the strength of this paradigm. Furthermore, we have also demonstrated that female animals show more time spent in the isolation zone regardless of the male partners sexual motivation state (Figure S2). From these results, we believe that this paradigm reports important metrics that correlate with female sexual motivation. However, it is also clear that other factors affect the females behavior in the self-paced mating paradigm. Recent studies developed an operant conditioning apparatus which delivers an intruder to a male subject in response to a nose poke (Minakuchi et al., 2024). This apparatus is a powerful approach to dissociate motivation and action which is difficult in commonly used self-paced paradigms. The self-paced mating paradigm used in our study has limitations to studying sexual motivation in detail. An advancement in the design of the apparatus may be essential to further study the neural circuitry that regulates female sexual motivation in fine detail.

Sensory representation of male ejaculation in the female brain and genitalia

Suppression of female sexual behavior and motivation after male ejaculation is not a unique phenomenon found in mice and can be observed in various insect species (Gillott, 2003). Studies in *Drosophila* has demonstrated that the primary trigger of this behavioral switch is the sex peptide (SP), a peptide released in the male seminal fluid (Chapman et al., 2003; Chen et al., 1988; Liu and Kubli, 2003). Studies in flies have further identified the receptor for SP as well as the neural circuitry which conveys the sensory information to the mating regulating neurons in the brain (Feng et al., 2014; Rezával et al., 2012; Yapici et al., 2008). In contrast to the well-studied system in *Drosophila*, little is known about how the mammalian brain senses male ejaculation. Histological studies in rats have shown that male ejaculation induces c-Fos expression widely in the brain, including the MPOA (Pfaus et al., 1993). Indeed, anesthesia to the female genitalia has been shown to disrupt pacing of mating, indicating that sensory input from the genitalia can impact female sexual behavior (Parada et al., 2014). Furthermore, artificial stimulation of the vagina or the clitoris of the female rat was further shown to induce c-Fos expression in the MPOA in a similar fashion as male ejaculation, indicating that at least tactile sensory input can be conveyed to this brain area (Parada et al., 2010; Pfaus et al., 1996). Ejaculation from vasectomized male animals were sufficient to suppress sexual behavior suggesting that at least chemical components from the testis is not necessary for this effect (Zhou et al., 2023). A recent study has shown that mechanical stretch induces serotonergic activity in the dorsal raphe, which resembles changes after male ejaculation, suggesting that stretch sensation is mediating male ejaculation signal to the brain (Troconis et al., 2023). Mouse genetics has also enabled identification of nerves innervating the genitalia (Qi et al., 2024). However, there are still to uncover on how stretch sensation in the genitalia is conveyed to the brain, and equally unclear how this information is ultimately transmitted to the MPOA. Our findings on how the male ejaculation signal is represented in the MPOA will be an ideal entry point to study the neural basis of genital-brain communication.

Appetitive behavior and male ejaculation ensembles in MPOA

The MPOA is a molecularly and functionally heterogenous brain region. One function MPOA has heavily been related to is parenting behavior. MPOA neurons which express *Gal* and/or *Calcr* has been demonstrated to regulate parenting behavior (Kohl et al., 2018; Wu et al., 2014; Yoshihara et al., 2021). From our data, neurons that respond to male ejaculation tended to co-express *Gal* and *Calcr* more than neurons that responded to appetitive behavior (**Figure S3**). However, we also found that chemogenetic activation of male ejaculation responsive MPOA neurons did not induce parenting behavior (**Figure S10K**). While it is necessary to conduct extensive analysis of how MPOA neurons respond to other behaviors, these results suggest that male ejaculation responding MPOA neurons may specifically regulate sexual behavior and motivation but not parenting behavior. A previous study also found that MPOA contains a subset of neurons which regulate female appetitive sexual behavior. MPOA neurons which express *Vgat*, *Esr1* and *Nts*, and projects to the ventral tegmental area (VTA) has been shown to respond to olfactory signals from the male mouse and to enhance female-to-male sniffing (McHenry et al., 2017). There is also a significant release of dopamine after the first female to male sniffing interaction as well as after male ejaculation (Dai et al., 2022). How the brain, especially the MPOA, increases sexual motivation before and during the appetitive phase, and how it suppresses motivation after male ejaculation, has been a long-lasting question in the field that is addressed throughout this work. The whole brain activity-dependent labeling experiment identified the MPOA as one of the brain regions which strongly increases Fos activity (**Figure 2**).

However, there were many other brain regions showing increased activity associated with male ejaculation, including ones that are not canonically involved in the regulation of social behavior. While it is possible that all these brain regions work in concert to suppress sexual motivation after

male ejaculation, our results demonstrate that a specific brain region, MPOA, is sufficient to evoke behavioral response. This suggests that all brain regions showing higher Fos activity are not necessarily causal to behavioral changes. It is also possible that these widespread brain regions may be a consequence of noise due to the long time-window in this method, which may be resolved by further development of genetic methods for neural ensemble labeling. In the MPOA, our *in vivo* calcium imaging data shows nearly half of the inhibitory neurons that positively respond to appetitive behavior also respond to male ejaculation (**Figure 4**). Our data also identified that there was no difference between the number of *Nts*+ cells activated by appetitive behavior and male ejaculation (**Figure S5**). These results suggest that there is a small but significant number of neurons which respond both to appetitive behavior and male ejaculation in the MPOA, and potentially regulate release of dopamine.

Although a large number of neurons that responded to female-to-male sniffing also respond to male ejaculation, the opposite is not true (**Figure 4**). Importantly, the chemogenetic activation of neurons that were active after male ejaculation suppressed the amount of sexual interaction in the self-paced mating assay, opposite to what was described when activating *Nts*+ MPOA neurons (McHenry et al., 2017). This suggests that there are at least two-distinct populations in the MPOA which regulate appetitive sexual behavior or sexual motivation in opposing directions.

Neural circuitry for female sexual behavior regulation

The neural circuitry that regulates female sexual behavior has been extensively studied in rodent models. From early lesion and electrical stimulation studies, it has been proposed that the rodent brain has a positive and negative regulatory system for female sexual behavior. The positive system has been proposed in rats, where Pfaff and Sakuma demonstrated that the lesion of the ventromedial hypothalamus (VMH) abolished sexual receptivity while electrical stimulation of the same region induces receptivity (Pfaff and Sakuma, 1979a, 1979b). Mouse genetics further showed that a subset of neurons in the ventrolateral part of the VMH is crucial for the regulation of female sexual behavior (Inoue et al., 2019; Yang et al., 2013). More specifically, *Esr1*+ *Cckar*+ (*Nts*-) cells in the VMHvl were found to be necessary and sufficient for female sexual receptivity (Liu et al., 2022; Yin et al., 2022). These results suggest that the VMHvl is the "command center" which positively regulate female sexual behavior.

The MPOA together with other brain regions has been proposed to form the negative regulating system. Lesion of the MPOA was shown to increase the sexual receptivity of the female rat and electrical stimulation was shown to reduce receptivity, suggesting that MPOA negatively regulates female sexual behavior (Pfaff and Sakuma, 1979b; Powers and Valenstein, 1972). In the current study, we demonstrate that chemogenetic activation of neurons in the MPOA which respond to male ejaculation is sufficient to suppress female sexual behavior and sexual motivation. This result suggests that these neurons in the MPOA constitute the negative regulating system. Importantly, both our single-cell calcium imaging results and histological analysis of the chemogenetic activation cohort suggest that the majority of these neurons are inhibitory (**Figure 3I** and **Figure S9G**). We further demonstrate that the inhibitory population contains neurons that show prolonged responses to male ejaculation which resemble the persistent change of sexual motivation. Other studies have also supported the idea that the inhibitory cell population is more related to the regulation of social behavior than the excitatory population (Fang et al., 2018; Hashikawa et al., 2021; Moffitt et al., 2018; Tsuneoka and Funato, 2021; G.-W. Zhang et al., 2021). Our results are also in line with this idea and suggest that the inhibitory neuronal population is the main regulator of the change in sexual motivation. The role of excitatory neurons that respond to male ejaculation will be a future topic to examine.

Recently, BNST was also found to play a role in negatively controlling female sexual behavior (Zhou et al., 2023). There, they use a behavioral assay which introduces 2 different males to the female subject before and after male ejaculation. They demonstrate that after male ejaculation, the female will be less receptive toward the second male. Importantly, this assay clearly

demonstrate that the reduction of female sexual behavior is due to the female herself, not due to the change in their male conspecific. This result together with the result from our self-paced mating assay (**Figure 1**) strongly indicates that the behavioral change after male ejaculation reflects change in internal motivational states. The study further used single-cell resolution *in vivo* calcium imaging techniques to demonstrate that $BNST^{Esr2}$ neurons show specific and robust responses after male ejaculation, like what we observed in the MPOA. They then use opto-and chemogenetic tools to study the causality of these neurons. They demonstrate that the inhibition of $BNST^{Esr2}$ neurons rescued female receptivity after male ejaculation, activation of these neurons did not affect sexual behavior in female mice, suggesting that $BNST^{Esr2}$ neurons are necessary but not sufficient to suppress female sexual behavior and sexual motivation. While there were some differences in how modulation of BNST and MPOA neurons affect sexual behavior and motivation, overall, the role of BNST and MPOA seems to be similar suggesting that the two regions work in concert to suppress female sexual behavior. This is contrary to the finding from Mei et al. showing that the $BNST^{Esr1}$ neurons and $MPOA^{Esr1}$ neurons forms a bidirectional inhibitory circuit to antagonistically regulate infanticidal behavior and maternal behavior respectively (Mei et al., 2023). A mechanism which is yet to be revealed can override the negative feedback loop between MPOA and BNST neurons, potentially a disinhibitory circuit that connects the $BNST^{Esr2}$ and $MPOA^{GABA}$, is necessary for these neurons to regulate the same behavior change.

Interestingly, a subpopulation in the VMHvl expressing Cckar show strong inhibition of spontaneous bulk calcium events after male ejaculation (Yin et al., 2022). This is opposite to the persistent increase of activity in the MPOA and BNST, suggesting that the activity in these regions is reflected onto the VMHvl. Indeed, MPOA and BNST both send dense projection to the VMHvl, and $VMHvl^{Esr1}$ neurons project to the MPOA and to the BNST, making the MPOA-BNST-VMHvl network highly interconnected (Dimén et al., 2021; Lo et al., 2019; Osakada et al., 2018). A recent study focusing on male aggression motivation further demonstrated that the long-range input from inhibitory MPOA neurons to VMHvl promote and prolongs low motivational state (Minakuchi et al., 2024). From this finding and the fact that the acute activation of MPOA neurons more drastically affected female sexual behavior than the $BNST^{Esr2}$ neurons, we speculate that the MPOA can directly inhibit the VMHvl, while the BNST utilizes a different circuit mechanism which either involves multiple synaptic connections or different molecular mechanisms to act on the VMHvl.

Long-lasting change in neural activity within this network, potentially through monoamine or steroid molecules such as dopamine or opioids may be the neural basis of suppressed sexual motivation after male ejaculation (Micevych and Meisel, 2017; S. X. Zhang et al., 2021). Another candidate is serotonin, which is known to be an important mediator for sexual behavior (Uphouse, 2014). Recent study has revealed release of serotonin in response to male ejaculation in female mice (Troconis et al., 2023). Our whole brain activity mapping data also indicates increased Fos activity in the dorsal raphe (**Figure S3 and S4**). Whether serotonin mediates the changes in persistent activity across the entire network and whether there is a difference in how it impacts MPOA and BNST and its output to the VMHvl will be an important topic of future studies.

Taken together, our study identifies a subset of neurons in the MPOA which is dedicated to negatively sustain female sexual motivation. This finding fills the missing hole in how the brain neurons regulate female sexual motivation and proposes that the basal forebrain complex of MPOA and BNST work in concert as the negative regulating system of female sexual behavior.

Materials and methods

Animals

All experiments were approved by the Institutional Animals Care and Use Committee at the University of Washington (Protocol #4450-01). Animals were group-housed with littermates on a 12-hr light cycle at ~22°C with food and water available *ad libitum*. Wild-type Swiss-Webster mice were purchased from Taconic Biosciences (NY, U.S.). Wild-type C57BL/6 mice were purchased from the Jackson laboratory and bred in-house. Vgat-Cre (also known as *Slc32a1*-ires-Cre, Jax#016962), Vglut2-Cre (also known as *Slc17a6*-ires-Cre, Jax#028863) and Ai14 (Rosa-CAG-LSL-tdTomato-WPRE, Jax#007914) were purchased from Jackson laboratory. TRAP2 mice (also known as Fos-2A-iCreER^{T2}, (DeNardo et al., 2019)) were provided by Dr. Palmiter (University of Washington) and crossed with Ai14 to generate TRAP2::Ai14 mice. All male mice which were used as intruders were singly housed to avoid male-male aggression. Female mice with GRIN-lens implant were singly housed. All other mice were group housed.

Surgery

For targeting AAV into a certain brain region, stereotactic coordinates were defined for each brain region based on the brain atlas (Franklin and Paxinos, 2007). Mice were anesthetized isoflurane and head-fixed to stereotactic equipment (Kopf). For *in vivo* calcium imaging experiments, 500 nL of AAVDj EF1a-DIO-GCaMP6s (2.8*e12, UNC viral core, lot#av78310) was injected into two coordinates in the target brain region at a speed of 60 nl/min using Nanoject 3 pump (Drummond Scientific). The following coordinates were used [Distance in millimeters from the Bregma for the anterior (A)-posterior (P) and lateral (L) positions, and from the brain surface for ventral (V) direction]: MPOA, A 0.2, L 0.15, V 5.2. and A 0.4, L 0.35, V 5.2. Immediately following viral injection, 5 screws were placed into the skull and a GRIN lens (Inscopix, 0.6 mm x 7.3 mm) was slowly lowered to position above the injection site: MPOA, A 0.3, L 0.3, V 4.95. GRIN lens was glued to the skull with C&B Metabond Kit (Parkell, # S380) and dental cement (Lang Dental, #1304CL). After placement of GRIN lens, a custom-made head ring was glued to the skull and screws. For bare GRIN lenses, more than 3 weeks after the virus injection and GRIN lens placement surgery, a baseplate (Inscopix) was positioned to while visualizing GCaMP6s expression with a microscope. Once a field of view with GCaMP6s expression was found, the baseplate was glued to the head ring and skull. For pharmacogenetic experiments, 300-400 nL of AAV5 hSyn-DIO-hM3D(Gq)-mCherry (2.0*e13, Addgene, #44361-AAV5, lot#v141469), AAV5 hSyn-DIO-hM4D(Gi)-mCherry (2.5*e13, Addgene, #44362-AAV5, lot#v178820) or AAV5 EF1a-DIO-eYFP (4.4*e12, UNC viral core, lot#av4802B) was bilaterally injected into the target brain region at a speed of 60 nl/min using Nanoject 3 pump. The following coordinates were used: MPOA, A 0.3, L 0.25, V 5.2. To avoid pregnancy during the first experience, all female mice that experienced a mating assay except ones used in **Figure 1 N–T** was ovariectomized. Ovariectomized female mice that were primed with hormones has been successfully used in other sexual behavior studies (Ring, 1944; Yang et al., 2013).Animals that were used *in vivo* calcium imaging experiments or pharmacogenetic experiments were ovariectomized during the virus injection or GRIN lens placement surgery. After surgery, the incision was sutured, and the animal was warmed using a heat pad to facilitate recovery from anesthesia.

Behavior experiments and analysis

For all mating assays, female mice older than 6 weeks were ovariectomized (OVX) under isoflurane anesthesia. If necessary, mice underwent surgeries for AAV injections, GRIN lens implantation on the same day. After more than 2 weeks of recovery, the OVX female subjects were subcutaneously (s.c.) injected with 0.1 mL and 0.05 mL Estradiol (E8515, Sigma-Aldrich, 0.1 mg/ml) dissolved in sesame oil (Sigma-Aldrich) at 24 and 48h before assay, and 0.05 mL Progesterone (P0130, Sigma-Aldrich, 10 mg/ml in sesame oil) at 4h before assay. If necessary, mice underwent I.P. injection of CNO/saline. C57BL/6 or Swiss-Webster mice were used as a stud male. A 4 – 6 cm to

head bar was placed onto the male mice under isoflurane anesthesia, which was used for the female self-paced mating assay described below. The male animals were kept isolated until the assay and had been trained prior to the assay to show mounting and ejaculation.

The home cage mating assay was conducted as previously described with modifications (Ishii et al., 2017). Each assay was conducted 5-10 hrs after the onset of dark period, under an IR light and recorded (30 Hz) for subsequent 30 to 120 min for analysis of sexual behavior from a dorsal view. For neural activation experiments using hM3Dq (**Figure S9**), 0.1 mL of 1 mg/kg clozapine-N-oxide dissolved in saline (CNO, RTI International, NIMH Code C-929) or 0.1 mL of saline was I.P. injected into the animal 30 minutes prior to the male entry. For neural inhibition experiments using hM4Di (**Figure S11**), 0.1 mL of 5 mg/kg CNO dissolved in saline or 0.1 mL of saline was I.P. injected into the animal 30 minutes prior to the male entry.

The female self-paced mating assay was conducted using a behavior apparatus modified from an apparatus used in rat (Nyuyki et al., 2011). A 1-cm thick black wall with a 20 mm x 20 mm hole 3D-printed with PFA filament was placed in a 25 cm x 35 cm cage to divide the room in a 1:2 ratio along the longer edge. The smaller area was defined as the isolation zone and the larger area was defined as the interaction zone. The female subjects that were not able to pass through the hole during the habituation was eliminated from the cohort. On the experiment day, the female subjects were placed into the behavior apparatus for 30 minutes for analysis of movement tracking from a dorsal view, which was used as control (Control trial). After the habituation, the partner (Swiss-Webster) was placed in the interaction zone. Each assay was conducted 5-10 hrs after the onset of dark period, under an IR light and recorded (15 Hz) for subsequent 30 to 120 min for analysis of movement tracking and sexual behavior from a dorsal view (Sexual interaction trial). For **Figure 6**, females underwent two mating assays at > 1 week interval, with different treatment (e.g., saline or CNO). Different males were used for each assay. For neural activation experiments using hM3Dq (**Figure 6**, **Figure S9**, **Figure S10**), 0.1 mL of 1 mg/kg CNO dissolved in saline or 0.1 mL of saline was I.P. injected into the animal 30 minutes prior to the male entry. For neural inhibition experiments using hM4Di (**Figure S11**), 0.1 mL of 5 mg/kg CNO dissolved in saline or 0.1 mL of saline was I.P. injected into the animal 30 minutes prior to the male entry.

Sexual behavior was annotated from the recorded video using Behavioral Observation Research Interactive Software (BORIS) in a frame-by-frame fashion (Friard and Gamba, 2016). Male mounts, intromission, male to female anogenital sniffing, female to male sniffing, female self-grooming and male ejaculation were annotated based on characteristic postures described previously (Ishii et al., 2017). Briefly, mount was defined as a stud male using both forepaws to climb onto a female from behind for copulation. Intromission was defined by stable thrusting from the male animal during mount. Male ejaculation was identified by increase and then termination of thrusting and more than 10 seconds of immobility from the male, which are postures frequently associated with male ejaculation. Male to female anogenital sniffing was identified by the male animal following the female subjects anogenital area. Female to male sniffing was identified by female subject nose approaching male subject. When analyzing sniff-evoked GCaMP activity, to restrict the analysis to appetitive sniffing behaviors, sniffing behaviors which happened after the first mounting were excluded from the analysis. Female self-grooming was identified by the female subject moving their head close to their genital region. The receptive ratio was calculated by dividing the total number of intromissions with the total number of mountings. Behavior annotation was conducted by volunteers who were blind for the stimulation or AAV condition. For the female self-paced mating assay, the position of the female subject was tracked from the recorded video using DeepLabCut (Nath et al., 2019) or Ethovision. The amount of time spent in the interaction zone was quantified using SimBA region of interest (ROI) interface (Nilson et al., 2020). Behavior data from *in vivo* calcium imaging experiments were down-sampled to 10-Hz to match the imaging data.

For conditional place preference (**Figure S10G–J**), a two chambered apparatus was used with visual (horizontal and vertical stripes) cues. The floor was backlit and an overhead video camera recorded position (Basler, 3.75 Hz frame rate with Ethovision software, Noldus). The apparatus was in a sound isolation chamber. *Ad libitum* fed Fos-CreER mice with hM3Dq and eYFP controls were acclimatized (60 min). The following day, the mouse's position was recorded (1,800 s) and tracked offline using Ethovision (pre-test). Daily conditioning consisted of two 1,800 s sessions: (1) one side of the chamber paired with saline injection; (2) the other side paired with CNO (1 mg/kg) injection. Saline or CNO was administrated 10 min prior to the entry to the chamber. The side on which saline or CNO was administered was counterbalanced. After three conditioning days, mice were given free access to the entire apparatus and their position was tracked (post-test). The difference of time spent in the CNO zone, the number of entries into the CNO zone and the velocity between the post-and pre-test.

For pup retrieval test (**Figure S10K–O**), a home cage of the female subject was used. Female subject was i.p. injected with saline as control or CNO in their home cage. 30-minutes after administration, three 7-day-old pups were placed in the corners of the home cage. Animal behavior was recorded from the dorsal view for 10 minutes. Parenting behavior was annotated from the recorded video using Behavioral Observation Research Interactive Software (BORIS) in a frame-by-frame fashion. Pup retrievals, nest building, pup grooming and pup sniffing were annotated based on characteristic postures. Briefly, pup retrieval was defined as a female subject carrying pup. Nest building was defined by female subject digging their bedding or carrying nesting material. Pup grooming was identified by grooming pups without carrying. Female to pup sniffing was identified by female subject nose approaching pup. For neural activation experiments using hM3Dq (**Figure S10 K–O**), 0.1 mL of 1 mg/kg CNO dissolved in saline or 0.1 mL of saline was I.P. injected into the animal 30 minutes prior to the pup introduction.

In vivo calcium imaging experiments and analysis

Miniature microscope nVoke 2.0 or nVue (Inscopix) was used to obtain GCaMP6s signal (20 Hz). The microscope was connected above the behavior apparatus using a commutator (Inscopix) to support the weight of the microscope. On the experiment day, the subject was habituated to the microscope for 10 minutes. After the habituation, the LED power and gain was adjusted to 0.2-0.5 and 6.0-8.0 respectively depending on the intensity of GCaMP6s expression. Data acquisition started after the adjustment of imaging parameters. The animal was kept habituated to the microscope for additional > 5 minutes to obtain background signal, and then the intruder male animal was introduced to the subject's home cage. Calcium activity as well as animal behavior was recorded until 2-10 minutes after male ejaculation. Imaging data were spatially down-sampled by factor of 4 and temporally down-sampled to 10 Hz using the Inscopix Data Processing software (1.8.0). The data was further filtered by a spatial band-pass filter and motion corrected using the same software. Masks were manually drawn to extract data from single cell. The data frame containing GCaMP6s signal intensity traces were exported from the Inscopix Data Processing software and further processed in Python. Signal intensity array was z-scored using the background signal. For peri event activity analysis in **Figure 3** and **Figure 4**, z-scored signal was collected from -5 to + 15 seconds around the behavior onset. Peri event activity from multiple behavior events were concatenated and averaged. Finally, the mean value of pre-behavior background (-5 to 0 seconds from onset) was subtracted. The magnitude of z-scored signal was quantified by averaging 0 to +5 seconds from onset. Cells that had larger than 2 d z-scored signal were classified as "responding" cells. For persistent activity analysis in **Figure 5**, z-scored signal was collected from -5 to + 120 seconds around the onset of male ejaculation. The mean value of pre-behavior background (-5 to 0 seconds from onset) was subtracted. The decay length was quantified by calculating the time the z-scored signal was smaller than 0. The magnitude of z-scored signal was quantified by averaging 0 to +5 seconds from onset. Spectral clustering was conducted using scikit-learn package and previously used custom script (Namboodiri et al., 2019). Briefly, first dimensionality reduction using principal component analysis was conducted for z-scored signal from -5 to +120 seconds around the onset of male ejaculation. Next, n = 4

principal components were used to project the data. This was further used as the input into the clustering algorithm while systematically testing nearest neighbor and number of cluster variables. The best variable that resulted in maximizing the silhouette score was determined (number of clusters = 5, number of nearest neighbors = 55, average silhouette score = 0.405). All clusters contained cells from multiple animal subjects, indicating the variability of the dataset can be explained by heterogenous cell population, rather than subject variability.

TRAP experiments

TRAP method was conducted as previously described with modifications (DeNardo et al., 2019). TRAP2::Ai14 or TRAP2 mice were OVX during isoflurane anesthesia and AAV injected if necessary. After 2 – 3 weeks from OVX surgery, animals were prepared for the activity labeling assay. Female subjects were subcutaneously (s.c.) injected with 0.1 mL and 0.05 mL Estradiol (E8515, Sigma-Aldrich, 0.1 mg/ml) dissolved in sesame oil (Sigma-Aldrich) at 24 and 48h before assay. On the day for the assay, female subjects were isolated in their home cage and introduced with a male mating partner to either experience appetitive behaviors, consummatory behaviors or male ejaculation. For the appetitive group, the male partner was removed after 10–20 minutes. To ensure that the female subject did not experience any consummatory behavior from the male, the male partner was removed whenever an attempt to mount was observed. For the consummatory group, the male partner was removed after the first male mounting and intromission was observed. For the post-ejaculatory group, the male partner was removed just after male ejaculation. Immediately after the removal of the male animal, the female subject was I.P. injected with 0.125 mL of 50 mg/kg of 4-Hydroxytamoxifen (Sigma-Aldrich, cat#H6278) dissolved in sesame oil. After the injection, the female subject was isolated in their home cage and kept in the behavior room for overnight to avoid background stimulation. On the following day, the mice returned to the regular housing.

HCR experiments and analysis

Hybridization chain reaction (HCR) was performed based on the protocol provided by Molecular Instruments with some modifications (**Figure S5** and **Figure S9D–G**). For *Fos* activity analysis (**Figure S5**), the female subject was first singly housed in the home cage for 1 hour. The male partner was introduced into the home cage and animal behavior was recorded. For the appetitive group, the male partner was removed after 10–20 minutes until they first attempt mounting to the female subject. For the completion group, the male partner was removed just after male ejaculation. Twenty-minutes after the removal of the male partner, the female subject was immediately sacrificed to collect brain tissue. The brain tissue was then snap-frozen using dry ice. Every fifth 20-micron coronal sections containing the MPOA (3 sections from Bregma +0.10 mm to – 0.10 mm) were collected on a slide glass and fixed with 4% PFA in PBS for 30 min and rinsed with PBS, followed by dehydration with a series of 50%, 70%, 100%, 100% of ethanol. After final dehydration the slides were dried. The tissue was treated with Protease4 (ACDBiosystem) for 5–7 minutes. The slides were then rinsed with PBS and were applied with hybridization buffer (Molecular Instruments) for 10 minutes at 37°C for pre-hybridization. RNA probes for *Fos, Vgat, Vglut2, Calcr, Esr1, Gal, Nts, Prlr* (1 mM, Molecular Instruments) were diluted in a ratio of 1:100–1:250 into the hybridization buffer. After pre-hybridization, hybridization buffer (70 µl) containing the probes was applied to each slide on which the parafilm cover was placed. After 12h–18h of incubation at 37°C in the moisture chamber, the sections were washed, with a series of probe wash buffer and SSC-T buffer (5x SSC, 0.1% Tween 20) mixture. After the final wash, the sections were incubated in amplification buffer (Molecular Instruments) for 30 minutes at room temperature for pre-amplification. Amplification hairpin probes (3 mM, Molecular Instruments) were diluted in a ratio of 1:50 into the hybridization and then snap cooled for 1.5 minutes in 95°C. After pre-amplification, amplification buffer containing hairpin probes (70 µl) was applied to each slide on which the parafilm cover was placed. After 12h–18h of incubation at room temperature in the moisture chamber, the sections were washed in 5xSSC-T buffer for 30 minutes x2. The sections were quenched for autofluorescence using Vector TrueView reagent (Vector, cat# SP-8400). After

washed in 2xSSC, the sections were stained with DAPI in PBS (5 µg/ml, Thermo Fisher Scientific, cat#D1306) for 8 minutes. The slide was then mounted using Vibrance Antifade Mounting Medium (VECTASHIELD, cat#H-1800). Images were obtained using a conventional microscope (Zeiss, Apotome Imager.M2) with a 20x objective. After image acquisition, the coverslip was removed and washed in 2x SSC for 10 minutes, then incubated with DNase1 (0.25 U/µl, Roche, cat#4716728001) for 1.5 h at room temperature. The section was then washed in 2x SSC for 5 minutes x6. The sections then proceeded to the pre-hybridization step for the next round of HCR. A total of 3 rounds of HCR were conducted. Images from multiple rounds were aligned using HiPlex image registration software (ACDBiosystem) or manually aligned using ImageJ package TrackEM2 (Cardona et al., 2012).

For anatomical characterization for TRAP2 animals used in **Figure S9D–G**, subjects were perfused with PBS and 4% PFA-PBS, then brain was harvested and post-fixed with PFA overnight. Every fourth 40-µm coronal sections containing the MPOA were collected on a slide glass. The sections went through 1 round of HCR and were stained for *Vgat* and *Vglut2* following the procedures described above.

The images with each probe signal were aligned to the DAPI channel to identify MPOA subregions and adjacent regions. The image was processed in HALO v3.2 software (Indica Labs) to segment each cell based on the DAPI signal and to quantify RNA transcript signal in each cell. Parameters for detecting puncta and intensity of each gene were manually adjusted for each tissue section of each animal by a volunteer blind to the experimental condition. The cutoff value to determine a gene “expressing cell” was defined as the mean fluorescent intensity of 3 to 5 transcripts depending on the background fluorescent signal. For supervised clustering of the MPN cell gene expression matrix (**Figure S5K–M**) was conducted using Seurat3.1.1 in R. Cells that had no mRNA signal were excluded from the dataset. This expression matrix contained single cells detected across all 10 animals and the spot counts for every gene; we also included signal intensity measurements as metadata. Once loaded, the HCR digital expression matrix was scaled but not normalized. During scaling, we regressed out gene intensity measurements on the basis that signal intensity may indicate staining quality. Next, cells were clustered using the same pipeline. The cells were UMAP plotted using nearest_neighbors = 100, minimum_distance = 0.05 (**Figure S5K**). The cluster ID for each cell was exported and used for statistical analysis using custom Python scripts.

Immunohistochemistry experiments

To evaluate the accuracy and efficiency of TRAP labeling, we utilized subjects used in pharmacogenetic experiments (**Figure 6**, **Figure S9**) and labeled neurons that respond to male ejaculation using immunohistochemistry against immediate early gene c-Fos. The TRAP2 female subjects were labeled with eYFP or hM3Dq-mCherry in the appetitive or male ejaculation responding neurons in the MPOA (appetitive-hM3Dq, consummatory-hM3Dq, post-ejaculatory-hM3Dq or post-ejaculatory-eYFP group). Ninety-minutes before perfusion, the female subjects experienced male ejaculation during a home cage mating assay (described in “Behavior experiments and analysis.”) to activate the male ejaculation responding neurons in the MPOA. The subjects were then perfused with PBS and 4% PFA-PBS, and the brain was harvested and post-fixed with PFA overnight. Every fourth 40-µm coronal sections containing the MPOA were collected. were washed three times with PBS-T (1x PBS, 0.3% Triton-X, Sigma-Aldrich, cat# 11332481001) for 10 min x3, and treated with 5% normal donkey serum (NDS, Sigma-Aldrich, cat# 566460) in PBST for 1 hr at room temperature for blocking. Sections were then incubated with rabbit anti-cFos antibody (Abcam, cat#190289-Rb, 1:500), diluted into 5% BSA in PBST for overnight at 4°C. Antibody signals were detected by goat anti-rabbit Alexa 488 or Alexa 594 (Invitrogen, cat#A21206 or cat#A21209, 1:500) for 2h at room temperature. Sections were washed once with PBST for 10 min, treated with PBS containing DAPI for 20 min, rinsed with PBS, and mounted with cover glass using Vibrance Antifade Mounting Medium (VECTASHIELD, cat#H-1800).

Sections were imaged by FV-3000 confocal microscope (Olympus) with a 20x objective. Images were processed in cellpose to segment DAPI positive cells in the MPOA and their subregions (Stringer et al., 2021). DAPI positive cell masks were used to quantify intensity of c-Fos protein or YFP/mCherry signal. The intensity value was used to determine the negative and positive cell.

Brain-wide analysis of activity-dependent labeling experiments

TRAP2::Ai14 females were used to label neurons responding to appetitive behavior or male ejaculation. Two-weeks after activity labeling (described in "TRAP experiments."), the animals were perfused with PBS and 4% PFA-PBS, the brain was harvested and post-fixed with PFA overnight. After post-fixation, the brains were processed for tissue clearing. For tissue clearing, we utilized stabilization under harsh conditions via intramolecular epoxide linkages to prevent degradation method (SHIELD) (Park et al., 2019). The procedures followed the SHIELD tissue clearing protocol (Full Active Pipeline Protocol v5.05, LifeCanvas). Briefly, the brain was incubated in SHIELD OFF solution [2.5 mL DI water, 2.5 mL SHIELD-Buffer Solution, 5 mL SHIELD-Epoxy Solution] at 4oC for 4 days. Then transferred to SHIELD ON buffer and incubated at 37°C for 24 hrs. Following, SHIELD ON buffer incubation, the brain was pre-incubated in Delipidization buffer for overnight at room temperature. The tissue was then placed into SmartClear II Pro (LifeCanvas) to conduct active tissue clearing for 24 hours. To match the refraction index, the tissue was incubated in 50% of EASYINDEX solution (RI = 1.52, LifeCanvas) at 37°C shaking for 24 hrs and then 100% of EASYINDEX solution at 37°C shaking for 24 hrs. The tissue was then embedded in EASYINDEX with 2% agar and mounted onto SmartSPIM light sheet microscope (LifeCanvas). The tissue was mounted on the ventral side facing toward the objective, imaged from the horizontal view using a 3.6x objective with 4-µm zstep. 594 nm channel was collected as the signal channel, 488 or 647 nm channel was collected as the background channel. Background channel was used for the alignment to the brain atlas. The images were first destriped and then stitched using a custom written script (LifeCanvas). The images were pre-processed and down sampled by 2 times (final resolution (x,y,z) = (3.6 µm, 3.6 µm, 4 µm)). Images were registered to a standard brain atlas Unified Anatomical Atlas (Yongsoo Kim Lab) (resolution (x,y,z) = (20 µm, 20 µm, 50 µm)) using ClearMap pipeline on a cloud-based cluster computer (Chon et al., 2019; Madangopal et al., 2022; Renier et al., 2016). tdTomato expressing cells were segmented through the same pipeline using a classifier created using Ilastik (Berg et al., 2019). To train the classifier, 10 chunks of images from the 4 of the subjects were randomly sampled and used as the training dataset (Image size, (x,y,z) = 1.8 mm x 1.8 mm x 100 µm). One chunk image of the same size from each subject was used as the test dataset. The quality of the classifier was visually examined. The total number of tdTomato positive cells per brain region was quantified and normalized by the size of each brain region (density, mm^3). In the standard brain atlas, brain regions were hierarchically sorted from broader regions to finer regions (level 1 to level 8). Level 4 was selected for broader analysis of brain regions (**Figure 2C**). Level 6 was selected for finer analysis of brain regions (**Figure 2H**, **Figure S4**). To compare the means of density of tdTomato cell in each brain area between the appetitive, consummatory and post-ejaculatory group, we conducted an ANOVA test and further corrected for multiple comparisons with Benjamini/Hochberg correction. False discovery rate (FDR) = 0.05 was used for the broader analysis while a loose discovery rate (FDR = 0.10) was used for finer analysis due to the large number of comparisons. A post-hoc Tukey's HSD post-hoc test (FWER = 0.05) was conducted for regions that tested significant in the ANOVA test.

Quantification, data processing and Statistical Analysis

Data were presented as mean with error bar of 95% confidence interval unless otherwise mentioned. Data processing and plot generation was done in Python and R. Plots were further processed in Adobe Illustrator to create Figures. The sample sizes were chosen based on previous studies using similar behavioral experiments. Codes used for analysis and statistics are provided on Github (*https://github.com/stuberlab/Ishii-Mating-completion.git*). The statistical details of each

experiment including the statistical tests used, exact value of n and what n represents, are shown in each figure legend. Each statistical test result is shown in Table 1. Significance was shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and not significant values were not noted or shown as n.s.

The sample size for behavior experiments in this study were n = 5-9. This was predetermined based on previous studies examining female sexual behavior (Ishii et al. 2017, Liu et al. 2022, Yin et al. 2022). To further examine the number of animals required for our behavioral experiments, we pooled data used in this study and conducted a power analysis (n = 111 pooled data, control n = 94, stim n = 17). We conducted a power analysis using the variance calculated from pooled average time in isolation zone. These data were pooled from control animals in each experiment (eg. animals with GFP control virus injected, saline injected, etc.). The average time in isolation zone in the after ejaculation or after reactivating the completion cells was 420 ± 210 seconds, and 49 ± 91 seconds in the control group (mean ± s.d.). Within this population, we found that 5 animals were sufficient to detect the difference ($p < 0.05$, power = 0.8) in Students t-test.

Data distribution

Whole brain FosTRAP activity mapping data is available on DANDI (*https://dandiarchive.org/dandiset/001249/0.241109.2357*). Other data used in this project is shared on Figshare (*https://doi.org/10.6084/m9.figshare.27642768.v1*). The code for analysis and data generation will be shared on GitHub (*https://github.com/stuberlab/Ishii-MPOA-Ejaculation-2023*).

Supplementary Figures

Figure S1

Sexual experience increase sexual motivation in female mice, related to Figure 1.

(A) Diagram showing the experiment design. Virgin animals were tested in a self-paced mating assay without any sexual experience. SE (sexually experienced) animals experienced male ejaculation in their home cage >7 days before the self-paced mating assay.
(B) The number of entries in the isolation zone per 10 minutes between virgin animals and sexually experienced animals. *p=0.041125541.
(C) The average time spent in the isolation zone between virgin animals and sexually experienced animals. *p=0.025974026.
All data are shown as mean ± 95% confidence interval and were analyzed by Mann-Whitney U test (**B** and **C**). Virgin: n = 6, SE: n = 6.
*p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

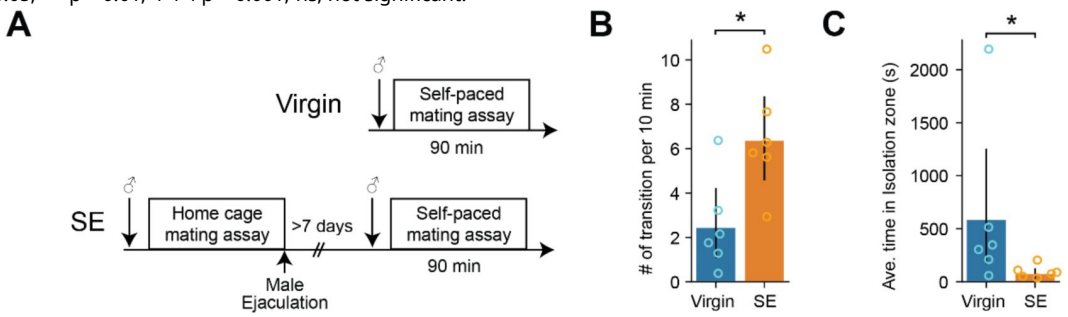
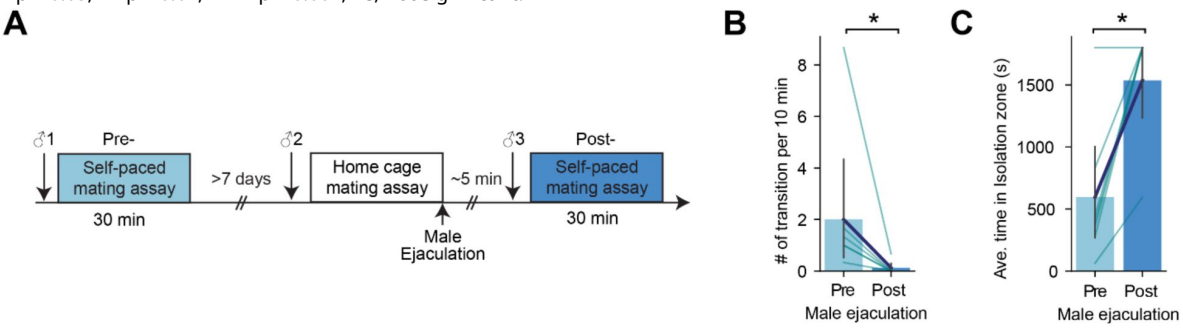


Figure S2

Reduction of sexual motivation after male ejaculation in female mice is not dependent on the state of the male mouse, related to Figure 1.

(A) Diagram showing the experiment design. First animals were put in a self-paced mating assay for 30 minutes (pre-ejaculation assay). After >7 days, the animals were put in a home cage mating assay until they experienced male ejaculation. Immediately after, they were put in a self-paced mating assay for 30 minutes (post-ejaculation assay). During each assay, the female mouse encountered a novel sexually active male mouse (male 1, 2 and 3).
(B) The number of entries in the isolation zone per 10 minutes between pre and post ejaculation assays. *p=0.027281171.
(C) The average time spent in the isolation zone between pre and post ejaculation assays. *p=0.027707849.
All data are shown as mean ± 95% confidence interval and were analyzed by Wilcoxon signed-rank test (**B** and **C**). n = 7.
*p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.



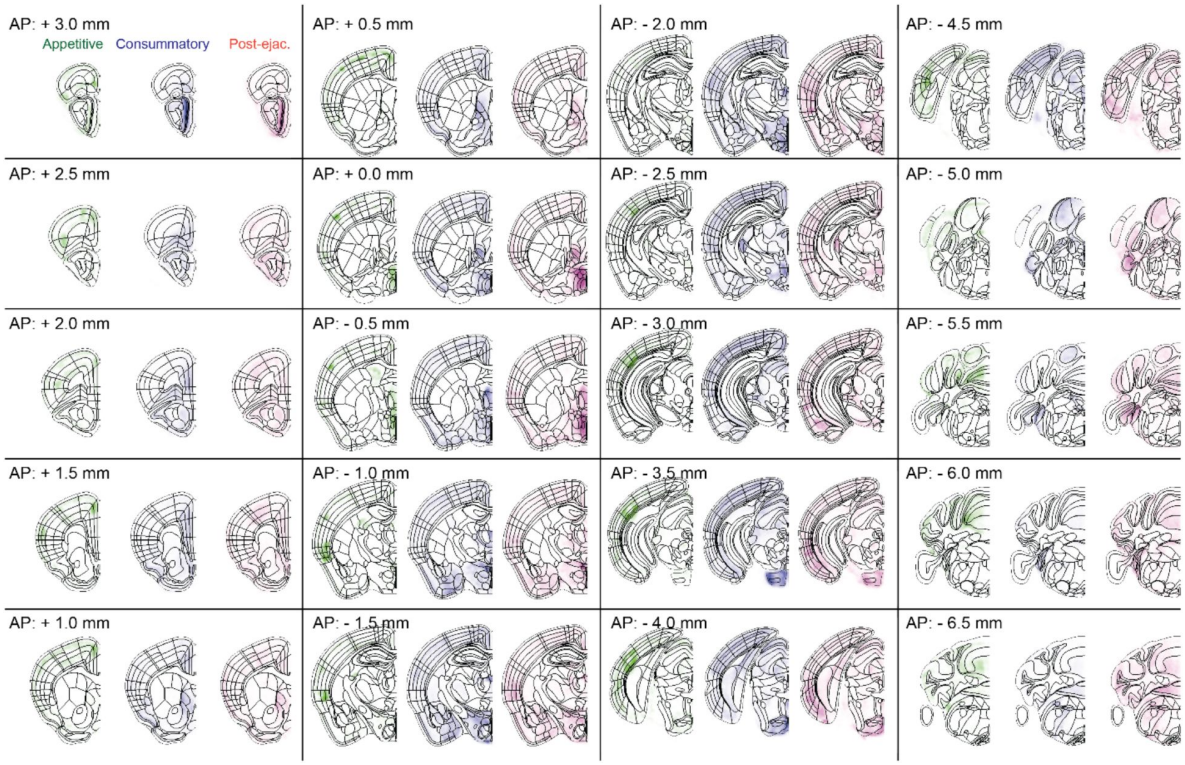


Figure S3

Brain-wide analysis of male ejaculation-responding cells in the female brain related to Figure 2 and Table 3.

Average density heatmap of tdTomato+ cells in the appetitive, consummatory and post-ejaculatory group. Appetitive, n = 4. Consummatory, n = 5. Post-ejaculator, n = 5.

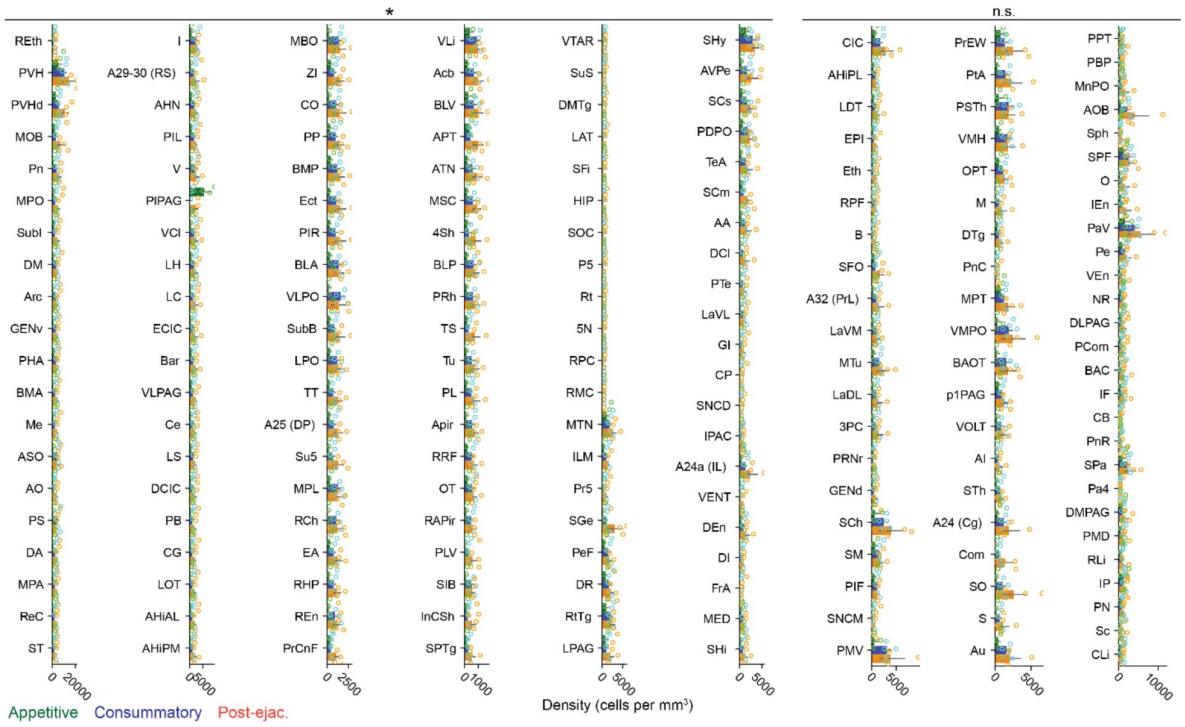


Figure S4

Brain-wide analysis of neurons active during post-ejaculatory period in the female brain related to Figure 2 and Table 3.

Density of tdTomato+ cells in each brain region from post-ejaculatory, consummatory and appetitive group. Statistical significances are shown above each column. The regions are sorted from the top left to bottom right by the qvalue and then the difference in the density between post-ejaculatory group and appetitive group. Details of statistical values and full name of acronyms are shown in Table 3. Appetitive, n = 4. Completion, n = 5. Post-ejaculatory, n = 5. ANOVA with Benjamini/Hochberg correction (FDR = 0.10), *q < 0.10; ns, not significant.

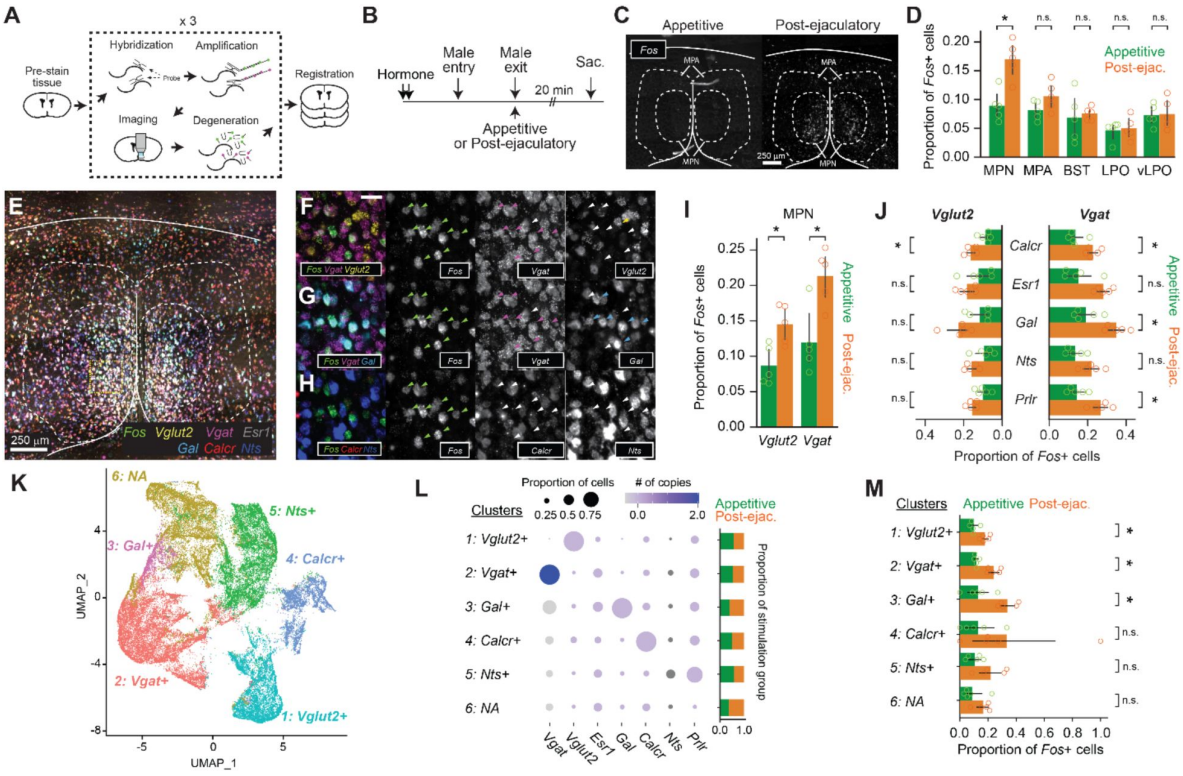


Figure S5

Male ejaculation responding cells are composed of a subset of excitatory and inhibitory cells in the MPOA.

(A) Brain sections went through multiplexed *in situ* RNA hybridization chain reaction (HCR) to identify cell type markers of MPOA and an immediate early gene *Fos. Vgat, Vglut2, Esr1, Gal, Calcr, Nts, Prlr* were used as cell type markers.
(B) Schematic of tissue sampling for *Fos* expression analysis in the MPOA. Wild type female subjects were administered with hormones before the experiment day. On the experiment day, male partners entered the female home cage. Male partners were removed immediately after ejaculation ("post-ejaculatory" group) or during the appetitive phase before they showed mounting ("appetitive" group). Brain tissue was harvested 20 minutes after the male exit.
(C) Representative coronal section showing *Fos* mRNA expression in the MPOA of Appetitive and Post-ejaculatory group.
(D) Proportion of *Fos*+ cells in each brain subregion. MPN: medial preoptic nucleus, MPA: medial preoptic area, BNST: bed nucleus of stria terminalis, LPO: lateral preoptic area, vLPO: ventral lateral preoptic area. BNST; t(8)=-0.369, p=1. LPO; t(8)=-0.3094, p=1. MPA; t(8)=-1.841, p=0.514. MPN; t(8)=-4.2709, *p=0.0136. VLPO; t(8)=-0.1751, p=1.
(E) Representative coronal section showing expression of 7 genes: *Fos, Vgat, Vglut2, Esr1, Gal, Calcr, Nts*.
(**F–H**) Expanded image of area highlighted in yellow in (E). (F) Expression of *Fos, Vgat* and *Slc17a6.* (**G**) Expression of *Fos, Vgat* and *Gal.* (H) Expression of *Fos, Calcr* and *Nts.* Colored arrow indicates cells expressing given gene. Scale bar, 50 μm.
(I) Proportion of *Vgat*+ or *Vglut2*+ and *Fos*+ cells in the MPN of Appetitive and post-ejaculatory group. *Vglut2*; t(8)=-3.3885, *p=0.019. *Vgat*; t(8)=-3.5361, *p=0.0153.
(J) Proportion of *Vgat*+ *Fos*+ or *Vglut2*+ *Fos*+ and cell type marker expressing cells in the MPN of Appetitive and post-ejaculatory group. *Vgat*+ Calcr+; t(8)=-3.6646, *p=0.0318. Vgat+ Esr1+; t(8)=-3.1442, p=0.0686. Vgat+ Gal+; t(8)=-4.0896, *p=0.0174. *Vgat*+ *Nts*+; t(8)=-2.6382, p=0.149. *Vgat*+ *Prlr*+; t(8)=-3.9487, *p=0.0212. *Vglut2*+ *Calcr*+; t(8)=-3.9487, *p=0.0146. *Vglut2*+ *Esr1*+; t(8)=-3.9487, p=0.8223. *Vglut2*+ *Gal*+; t(8)=-3.9487, p=0.1299. *Vglut2*+ *Nts*+; t(8)=-3.9487, p=0.1951. *Vglut2*+ *Prlr*+; t(8)=-3.9487, p=0.1038.
(K) UMAP plot of MPN cells.
(L) Disc plot showing proportion of gene expressing cell and the number of copies per cell for each gene and cluster. The proportion of cells from each group are shown on the right. Cluster *Vglut2*+; t(8)=-4.1631, *p=0.0189. Cluster *Vgat*+; t(8)=-5.9018, **p=0.0022. Cluster *Gal*+; t(8)=-3.9208, *p=0.0265. Cluster *Calcr*+; t(8)=-1.5438, p=0.9993. Cluster *Nts*+; t(8)=-2.1595, p=0.377. Cluster N.A.; t(8)=-1.8254, p=0.6322.
(M) Proportion of *Fos*+ cells from Appetitive and post-ejaculatory group in each cluster.
All data are shown as mean ± 95% confidence interval and were analyzed by Student's t-test with Bonferroni correction (**D, E, K** and **N**). appetitive group, n = 5. post-ejaculatory group, n = 5. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

Figure S6

Lens placement and field of view of *in vivo* imaging experiment related to Figure 3, Figure 4, Figure 5.

(A) Schematic illustration showing position of GRIN lens placement from each animal. Positions shown on the left hemisphere are from Vglut2 animals, the ones on the right hemisphere are from Vgat animals.
(B) Field of view and region of interest (ROI) for each animal. The subject ID and the number of ROI in each FOV are shown above. Vgat-Cre: n = 6, Vglut2-Cre: n = 4.

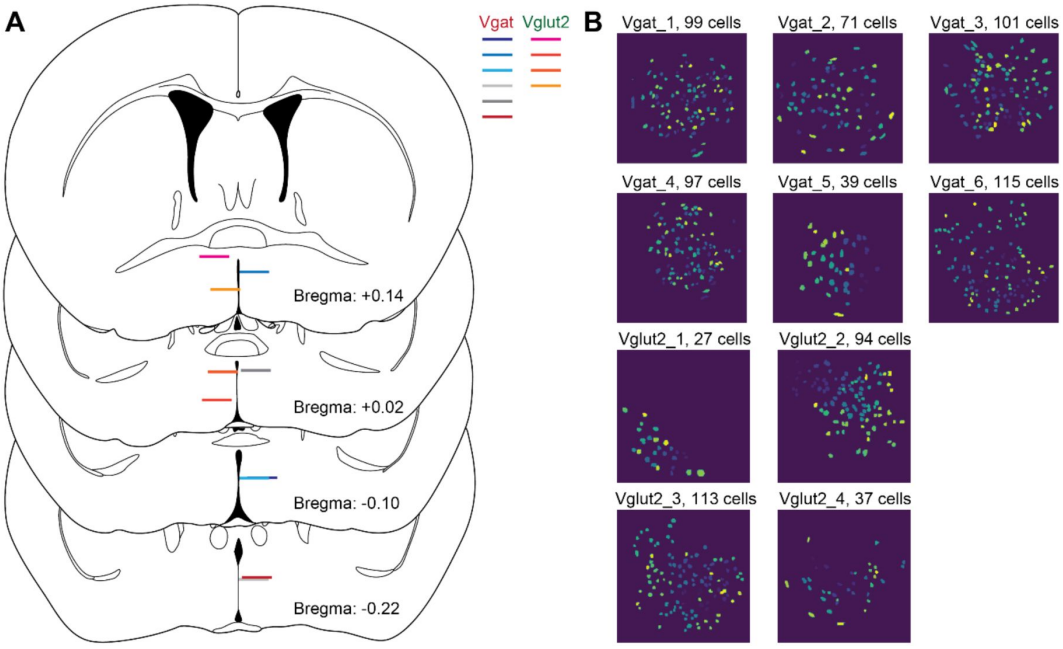


Figure S7

Distribution of subjects per cluster related to Figure 4.

Cells that had significant positive or negative response (>28) to each behavior onset were categorized as positive-or negative-responding cells respectively. Outer ring indicates the mouse line Vgat or Vglut2. The inner ring indicates each subject. Vgat-Cre: n = 6, Vglut2-Cre: n = 4.

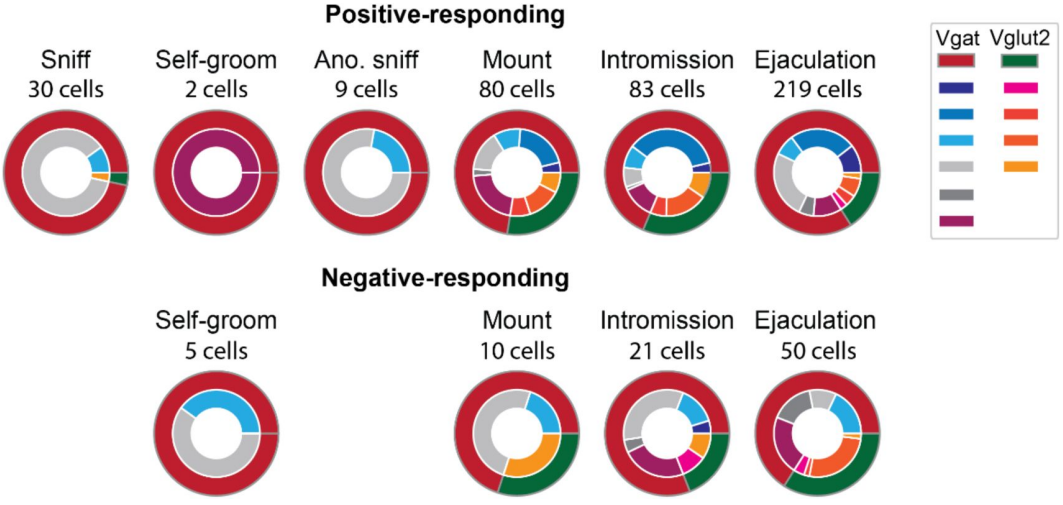


Figure S8

Distribution of subjects per cluster related to Figure 5.

Cells that had significant positive or negative response (>28) to each behavior onset were categorized as positive-or negative-responding cells respectively. Outer ring indicates the mouse line Vgat or Vglut2. The inner ring indicates each subject. Vgat-Cre: n = 6, Vglut2-Cre: n = 4.

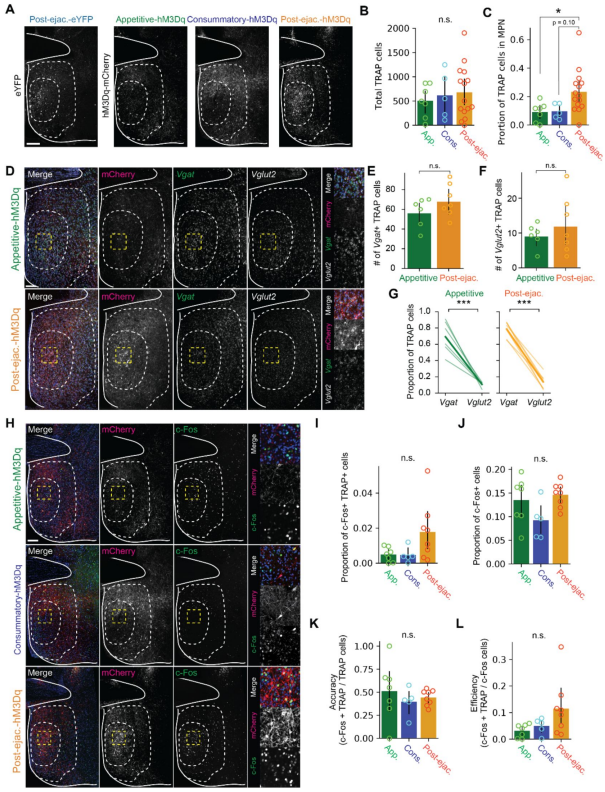


Figure S9

Histological analysis of TRAP labeling related to Figure 6.

(A) Histological analysis of the distribution of TRAP labeled cells. Representative coronal section showing eYFP or hM3Dq-mCherry expression in the MPOA. Scale bar, 100 μm.
(**B–C**) Data from post-ejaculatory-eYFP and post-ejaculatory-hM3Dq were grouped into post-ejaculatory group. Appetitive group, n = 7. Consummatory group, n = 5. post-ejaculatory group, n = 14.
(B) Number of TRAP labeled cells. One-way ANOVA, F(2,24)=0.3072, p=0.7383.
(C) The proportion of TRAP labeled cells in the MPN. One-way ANOVA, F(2,24)=4.3335, *p=0.0247. Tukey HSD; Appetitive vs Consummatory, p=0.9975. Appetitive vs Post-ejaculatory, *p=0.0471. Consummatory vs Post-ejaculatory, p=0.0984.
(D) *In situ* RNA hybridization analysis of the excitatory and inhibitory marker gene expression in the MPN. appetitive-hM3Dq and post-ejaculatory-hM3Dq groups were used for analysis. Representative coronal section showing mCherry, *Vgat* and *Vglut2* expression in the MPOA. Enlarged image indicated by the yellow box, is shown on the right.
(**E–G**) Number of *Vgat*+ TRAP labeled cells in the MPN (**E,** t(11)=-1.3299, p=0.2105), number of *Vglut2*+ TRAP labeled cells in the MPN (**F**, t(11)=-0.7917, p=0.4453.) and the proportion of TRAP labeled cells that express *Vgat* or *Vglut2* (**G,** Appetitive, t(10)=6.6154, **p=0.0024. Post-ejaculatory; t(12)=13.9477, ***p=1.69E-05). Appetitive group, n = 6. Post-ejaculatory group, n = 7.
(**H**) Antibody analysis of c-Fos expression after reactivation of male ejaculation ensemble. Representative coronal section showing mCherry and c-Fos expression in the MPOA. Enlarged image indicated by the yellow box, is shown on the right.
(**I–L**) proportion of c-Fos+ and TRAP labeled cells in the MPN (**I,** One-way ANOVA, F(2,17) = 3.2569, p=0.0635. Tukey HSD; Appetitive vs Consummatory, p=0.9991. Appetitive vs Post-ejaculatory, p=0.0942. Consummatory vs Post-ejaculatory, p=0.1268.), proportion of c-Fos+ cells in the MPN (**J,** One-way ANOVA, F(2,17)=3.1285, p=0.0697. Tukey HSD; Appetitive vs Consummatory, p=0.1724. Appetitive vs Post-ejaculatory, p=0.8445. Consummatory vs Post-ejaculatory, p=0.0631), the accuracy score (proportion of c-Fos+ TRAP labeled cells among all TRAP labeled cells) (**K,** One-way ANOVA, F(2,17)=0.4666, p=0.6349) and the efficiency score (proportion of c-Fos+ TRAP labeled cells among all c-Fos+ cells) (**L,** One-way ANOVA, F(2,17)=2.8313, p=0.0868. Tukey HSD; Appetitive vs Consummatory, p=0.9028. Appetitive vs Post-ejaculatory, p=0.0882. Consummatory vs Post-ejaculatory, p=0.2666). Appetitive group, n = 6. Consummatory group, n = 5. Post-ejaculatory group, n = 8.
All data are shown by thin lines for individual subjects and thick lines for mean. The data were analyzed by ANOVA test with Tukey's HSD post-hoc test (FWER = 0.05) (**B, C, I, J, K, L**), Student's t-test (**E**, **F**) or Student's relative t-test (**H**). *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

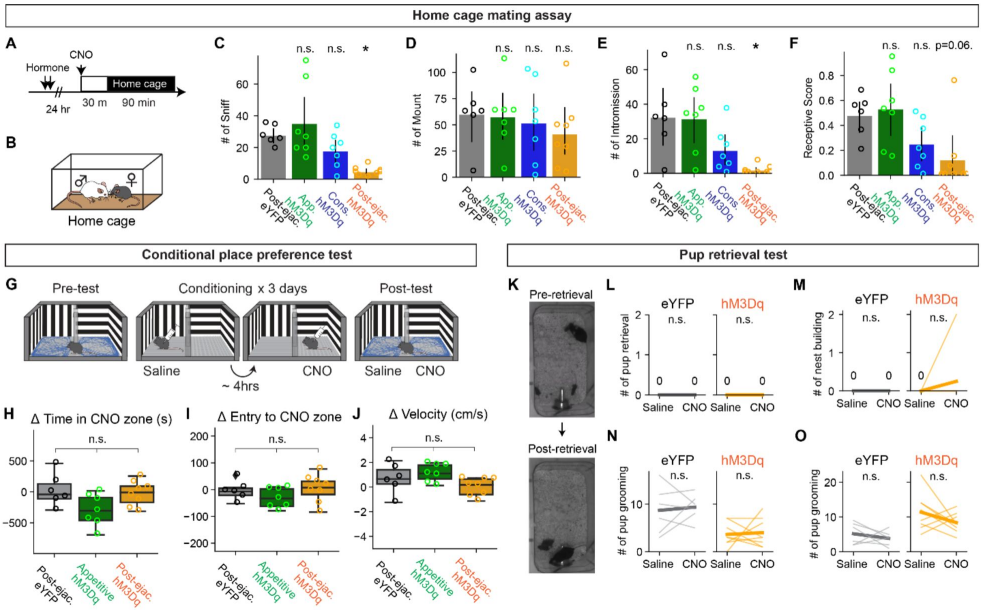


Figure S10

MPOA$^{post\text{-}ejaculatory}$ cells supress female sexual behavior in home cage mating assay but not other behaviors, related to Figure 6.

(**A**) Schematic of pharmaco-genetic activation of MPOA neurons during a home cage mating assay. Female subjects were primed with hormone before the experiment day. On the experiment day, subjects were injected i.p. with CNO to activate TRAP cells 30 minutes before the experiment. Animal behavior was recorded for 90 minutes.
(**B**) The male partner was placed into the home cage of the female subject.
(**C–F**) Number of female-to-male sniff (**C,** Post-ejaculatory-eYFP vs Appetitive-hM3Dq, p=1. vs Consummatory-hM3Dq, p=0.2581. vs Post-ejaculatory-hM3Dq, **p=0.0072.), male mounting (**D,** Post-ejaculatory-eYFP vs Appetitive-hM3Dq, p=1. Post-ejaculatory-eYFP vs Consummatory-hM3Dq, p=1. Post-ejaculatory-eYFP vs Post-ejaculatory-hM3Dq, p=0.4116.), male intromission (**E,** Post-ejaculatory-eYFP vs Appetitive-hM3Dq, p=1. Post-ejaculatory-eYFP vs Consummatory-hM3Dq, p=0.3042. Post-ejaculatory-eYFP vs Post-ejaculatory-hM3Dq, *p=0.0165) and the receptive score (number of mounting episodes with intromissions over all mounting episodes) (**F**, Post-ejaculatory-eYFP vs Appetitive-hM3Dq, p=1. Post-ejaculatory-eYFP vs Consummatory-hM3Dq, p=0.1888. Post-ejaculatory-eYFP vs Post-ejaculatory-hM3Dq, p=0.0626).
(**G**) Schematic of conditional place preference (CPP) assay to evaluate the valence of activation of MPOA neurons. A two-chamber behavior apparatus, one room with vertical stripes and one room with horizontal stripes. On the first day animals were placed in the behavior apparatus to test their initial preference to each room for 30 minutes (Pre-test). On the conditioning days, the animals were placed in one side of the room where they were i.p. injected with either saline as control or CNO. CNO was injected 4 hours after the saline injection. On the last day, the animals were placed in the behavior apparatus to test their preference 30 minutes (Post-test). (**H–J**) The difference in the time spent in the CNO zone (Time spent in the CNO zone in Post-test – Pre-test) (**H,** Post-ejaculatory-eYFP vs Appetitive-hM3Dq, p=0.2203. Post-ejaculatory-eYFP vs Post-ejaculatory-hM3Dq, p=1. Appetitive-hM3Dq vs Post-ejaculatory-hM3Dq, p=1.), the difference in the number of CNO zone entries (Entries in Post-test – Pre-test) (**I,** Post-ejaculatory-eYFP vs Appetitive-hM3Dq, p=1. Post-ejaculatory-eYFP vs Post-ejaculatory-hM3Dq, p=1. Appetitive-hM3Dq vs Post-ejaculatory-hM3Dq, p=1.) and the difference in velocity of the animal (Velocity in Post-test – Pre-test) (**J,** Post-ejaculatory-eYFP vs Appetitive-hM3Dq, p=1. Post-ejaculatory-eYFP vs Post-ejaculatory-hM3Dq, p=0.8472. Appetitive-hM3Dq vs Post-ejaculatory-hM3Dq, p=0.8472.).
(**K**) Representative image of pup retrieval test. Female subject was i.p. injected with saline as control or CNO in their home cage. 30-minutes after injection, 3x 7w-old pup was placed in the corner of the home cage. Animal behavior was examined for 10 minutes.
(**L–O**) The number of pup retrievals (**L,** eYFP, p=1, hM3Dq, p=1), the number of nest building (**M,** eYFP, p=1, hM3Dq, p=0.635), the number of pup grooming (**N,** eYFP, p=1, hM3Dq, p=1) and the number of pup sniffing (**O,** eYFP, p=1, hM3Dq, p=0.391).
All data are shown by thin lines for individual subjects and thick lines for mean. Appetitive-hM3Dq, consummatory-hM3Dq and post-ejaculatory-hM3Dq group were tested against post-ejaculatory-eYFP group by Mann–Whitney U test with Bonferroni correction (**C–F**, **H–J**) and Wilcoxon rank sum test (**L–O**) with Bonferroni correction. post-ejaculatory-eYFP: n = 6, appetitive-hM3Dq: n = 7, consummatory-hM3Dq: n = 7. post-ejaculatory-hM3Dq: n = 8. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not significant.

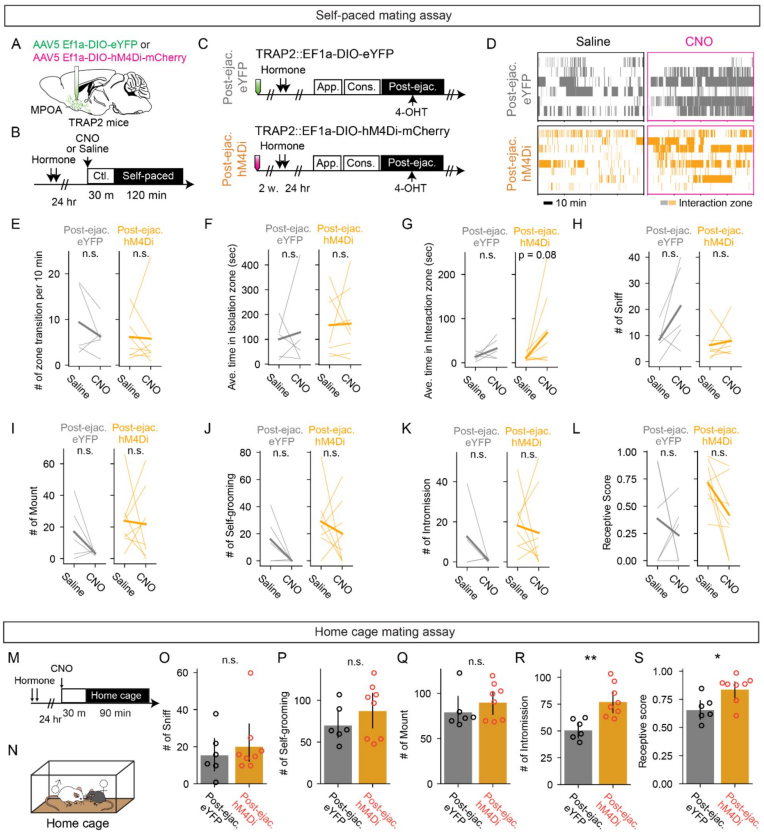


Figure S11

MPOA$^{post\text{-}ejaculatory}$ cells is not necessary for sexual motivation but for sexual receptivitiy.

(A) Schematic of selective labeling of MPOA neurons using TRAP2. AAV5 EF1a-DIO-hM4Di-mCherry or AAV5 EF1a-DIO-eYFP as control was injected into the MPOA of the female TRAP2 subjects. After incubation of the virus, the female subjects went through procedures to TRAP cells in MPOA (further described in **C**).
(B) Schematic of pharmaco-genetic inhibition of MPOA neurons during a female self-paced mating assay. Female subjects were primed with hormone before the experiment day. On the experiment day, subjects were injected i.p. with CNO to inhibit TRAP cells, or saline as control. 30 minutes after the injection, the male partner was placed into the apparatus and the animal behavior was recorded for 2 hrs.
(C) After incubation of the virus, the female subjects were administered with hormones, then received an ejaculation from the male partner. Immediately after, the subjects were administrated with 4-hydroxytamoxifen (4-OHT).
(D) Raster plots of time spent in the interaction zone the female self-paced mating assay (Post-ejaculatory-eYFP: gray. Post-ejaculatory-hM3Dq: orange).
(**E–L**) Number of zone transitions per 10 minutes (**E**, eYFP, p=0.4375. hM4Di, p=1.), average time in isolation zone (**F**, eYFP, p=1. hM4Di, p=1.), average time in interaction zone (**G**, eYFP, p=0.3125. hM4Di, p=0.15625.), number of female-to-male sniff (**H**, eYFP, p=0.1592. hM4Di, p=0.9917.), female self-grooming (**I**, eYFP, p=0.2883. hM4Di, p=1.), male mount (**J**, eYFP, p=0.4456. hM4Di, p=1.), male intromission (**K**, eYFP, p=0.4456. hM4Di, p=1.) and the receptive score (number of mount episodes with intromissions/number of all mount episodes) (**L**, eYFP, p=1. hM4Di, p=0.1094).
(M) Schematic of pharmaco-genetic inhibition of MPOA neurons during a home cage mating assay. Female subjects were primed with hormone before the experiment day. On the experiment day, subjects were injected i.p. with CNO to activate TRAP cells 30 minutes before the experiment. Animal behavior was recorded for 90 minutes.
(N) The male mating partner was placed into the home cage of the female subject.
(**O–R**) Number of female-to-male sniff (**O**, p=0.6969.), male mounting (**P**, p=0.4003.), male intromission (**Q,** **p=0.0045.) and the receptive score (number of mounting episodes with intromissions over all mounting episodes) (**R**, *p=0.01998.).
All data are shown by thin lines for individual subjects and thick lines for mean. The data were analyzed by Wilcoxon rank sum test (**E– L**) with Bonferroni correction. Post-ejaculatory-eYFP: n = 6, post-ejaculatory -hM4Di: n = 8, or by Mann-Whitney U test (**O–R**). Post-ejaculatory -eYFP: n = 6, post-ejaculatory -hM4Di: n = 8 $^{*}p < 0.05$, $^{**}p < 0.01$, $***p < 0.001$; ns, not significant.

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Additional information

Author Contributions

Experiments were conceived and designed by K.K.I. and G.D.S. G.D.S. supervised the project, provided resources, and funding. K.H. provided transcriptional analysis data of the MPOA and assisted in gene selection of HCR experiments. J.C. conducted HCR experiments and histological analysis. S.Y., R.E.F., S.K. and M.S. conducted behavioral experiments as well as behavioral annotations. C.Z. provided *in vivo* imaging data analysis pipelines. J.N., A.D.M, E.R.S., and S.A.G. assisted and supervised tissue clearing experiments and whole-brain data analysis.

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Reviewer #1 (Public review):

Summary:

This manuscript by Ishii et al utilize a classical, but extremely understudied, female self-paced assay to directly address aspects of female sxual motivation independent from the male's behavior. This allowed for clear separation of appetitive and consummatory events, of which whole brain unbiased activity was mapped. Mating completion in females was then focused to the medial preoptic nucleus where the authors performed a rigorous set of single-cell GCaMP recordings in populations marked by Vglut2 and Vgat, finding the latter display stronger and prolonged activity after the onset of mating completion. Finally, they demonstrate function to these Fos-TRAPPED completion cells demonstrating their capacity to suppress female sexual behavior.

Strengths:

This manuscript sought to explicitly explore female mating drive as dictated by the female, a very rare angle for those studying mating behavior which almost always is controlled by the male's behavior. To achieve this, the authors went back to old literature and modified a classical paradigm in which measurable approach and avoidance of male conspecifics can be measured in female mice using a self-paced mating assay. Strengths include a detailed quantification of female behaviors demonstrating a robust attenuated sexual motivation in females after mating completion. To determine the neural basis behind this, a brain wide analysis of cells responding to mating completion in the female brain was conducted which revealed numerous anatomical regions displaying increased Fos activity, including the MPOA, of which the authors concentrated the remaining of their study. Employing microendoscopic imaging, the authors discovered that this mating completion signal was strongly represented in the MPOA. The single cell data analyses are of very high quality as is the number of individual cells resolved. While they identified both excitatory and inhibitory cell types that were activated by mating completion, they found the latter exhibited stronger and more persistent activity. Segmentation into individual mating behaviors reinforced the importance of GABAergic completion cells, which display prolonged activity late after the onset of mating completion. This information provides a potential mechanism for how female mice suppress further mating activity following completion. The authors then definitively demonstrate this function by TRAP'ping completion cells with chemogenetic actuators and show that CNO-induced activation of these cells specifically and strongly suppresses female sexul behavior. All experiments were extremely well-designed and performed carefully and expertly with the necessary controls solidifying the conclusions.

Weaknesses:

While there are no glaring weaknesses in this study, it should be noted that a great deal of literature has pinpointed the MPOA (and specifically inhibitory cells in this area) as being critical to sexual behavior, including female mating. However, no study to my knowledge has explored self-paced female mating with such fine control over manipulating and monitoring cellular activity in this region. In addition, this study may act to inspire others to further explore the additional brain regions found to show upregulation of neural activity (Fos) during mating completion in the female using the data sets generated here.

Comments on revisions: The data has been provided in a public database.

https://doi.org/10.7554/eLife.91765.2.sa3

Reviewer #2 (Public review):

Summary:

In this set of studies, authors identify cFos activation in neurons in female mice that mated with males, and after experiencing male sexual behavior that is either restricted to appetitive behavior or including ejaculation. The medial preoptic nucleus was identified as an area with high cFos induction following ejaculation. Characterization of neurochemical phenotypes of cfos-expressing neurons showed a heterogenous distribution of activated neurons in the MPOA, including both inhibitory and excitatory cell types. Next, in vivo calcium imaging was used to show activation of Vgat and Vglut neurons in female mice MPOA after displaying sniffing of the male, experiencing male appetitive, or male consummatory sexual behavior, demonstrating significantly higher activation and of a greater subpopulation of Vgat neurons than Vglut neurons. Moreover, greatest activation of Vgat neurons was detected following experiencing ejaculation, and ejaculation activated different subpopulations of MPOA cells than consummatory or appetitive sexual behaviors experienced by the female. Finally, pharmaco-genetic activation of the subpopulation of MPOA neurons that were previously activated following ejaculation resulted in a significant reduction of approach behavior by the female mice towards the male, interpreted as suppression of female sexual motivation. In conclusion, a subpopulation of inhibitory cells in the MPOA is activated in female mice after experiencing ejaculation, in turn contributing to suppression of sexual approach behavior.

Strengths:

The current set of studies replicates previous findings that ejaculation causes longer latencies to initiate interactions with a male after receiving an ejaculation in a paced mating paradigm, which is widely validated and extensively used to investigate sexual behavior in female rodents. Studies also confirm that ejaculation increases cFos expression in the MPOA, while extending prior findings with a careful analysis of the neurochemical phenotype of activated neurons. A major strength of the studies is the use of cell-specific in vivo imaging and pharmaco-genetic activation to reveal a functional role of specific neuronal ensemble within the MPOA for post ejaculatory female sexual behavior.

Weaknesses:

The authors include an elegant manipulation of ejaculation-activated neurons in the MPOA using DREADD. However, this study was limited to show that activation of previously activated cells was sufficient to reduce approach behavior in a paced mating paradigm and receiving intromissions in a home cage mating paradigm. An inhibition approach using DREADD would have been a great complement to this study as it would have examined if activation of the cells was required. Moreover, additional tests for sexual motivation would have greatly strengthened the overall conclusions.

https://doi.org/10.7554/eLife.91765.2.sa2

Reviewer #3 (Public review):

Summary:

Ishii et al used molecular genetics, behavioral analyses, in vivo neural activity imaging, and neural activity manipulations in mice to study the functional role of a subset of medial preoptic area (MPOA) neurons in the regulation of female sexual drive. They first employed a self-paced mating assay during which a female could control the amount of interaction time with a male to assess female sexual drive after completion of mating. The authors observed that after mating completion (i.e., male ejaculation) females spend significantly less time interacting with males, indicating that their sexual drive is reduced. Next, the authors performed a brain-wide analysis of neurons activated following male ejaculation and identified the MPOA as a strong candidate region. One caveat is that the activity labeling was not exclusive to neurons activated following male ejaculation but included all neurons activated before, during, and after the mating encounter. However, in this revised version of the manuscript, the authors have included a key control group that labels all neurons activated up to but not including male ejaculation. Comparison of the number of activated neurons in these two groups revealed a significant additional set of neurons in the female MPOA following ejaculation. Importantly, the authors also provided in vivo calcium imaging data showing that a subset of MPOA neurons responds significantly and specifically to male ejaculation and not other behaviors during the social encounter. The authors performed these studies in both excitatory and inhibitory populations of the MPOA. Their analysis identified a subpopulation of inhibitory neurons that exhibit sustained increased activity for 90 sec following male ejaculation. Finally, the authors used chemogenetics to activate MPOA neurons during home cage mating, condition place preference, pup retrieval, and the self-paced mating assay. They found that activation of female MPOA neurons that were previously activated following male ejaculation significantly reduces mating behaviors and time spent interacting with a male during the self-paced mating assay. Whereas, activation of female MPOA neurons that were previously activated during consummatory behaviors but not male ejaculation does not alter mating behaviors and time spent interacting with a male. Therefore, MPOA neurons activated following ejaculation are sufficient to suppress female sexual motivation.

The authors' experimental execution is rigorous and well performed. Their data identify inhibitory neurons in the female MPOA as a neural locus that is activated following male ejaculation and whose prolonged activity plays a key role in the regulation of female sexual motivation. The addition of some key control groups to this revised version of the manuscript greatly strengthens the interpretation of the authors' findings.

Strengths:

(1) The use of the self-paced mating assay in combination with neural imaging and manipulation to assess female sexual drive is innovative. The authors correctly assert that relatively little is known about how male ejaculation affects sexual motivation in females as compared to males. Therefore, the data collected from these studies is important and valuable.

(2) The authors provide convincing histological data and analyses to verify and validate their brain-wide activity labeling, neural imaging, and chemogenetic studies.

(3) The single cell in vivo calcium imaging data are well performed and analyzed. They provide key insights into the activity profiles of both excitatory and inhibitory neurons in the female MPOA during mating encounters. The authors identification of an inhibitory

subpopulation of female MPOA neurons that is selectively activated following completion of mating is fundamental for future experiments which could potentially find a molecular marker for this population and specifically manipulate these neurons to understand their role in female sexual motivation in greater detail.

(4) The authors provide convincing evidence that activation of female MPOA neurons activated following male ejaculation is sufficient to suppress female sexual motivation. Importantly, the authors addition of the consummatory-hM3Dq group demonstrates that activation of female MPOA neurons activated during mating behaviors prior to male ejaculation is not sufficient to suppress female sexual motivation.

Weaknesses:

In this revised version of the manuscript, the authors have added important controls as well as additional clarifying text that adequately address the weaknesses that were present in the original version of the manuscript.

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Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *[…] Weaknesses:*
>
> *While there are no glaring weaknesses in this study, it should be noted that a great deal of literature has pinpointed the MPOA (and specifically inhibitory cells in this area) as being critical to sexual behavior, including female mating. However, no study to my knowledge has explored self-paced female mating with such fine control over manipulating and monitoring cellular activity in this region. In addition, this study may act to inspire others to further explore the additional brain regions found to show upregulation of neural activity (Fos) during mating completion in the female using the data sets generated here.*
>
> ***Reviewer #2 (Public Review):***
>
> *[…] Weaknesses:*
>
> *The authors include an elegant manipulation of ejaculation-activated neurons in the MPOA using DREADD. However, this study was limited to show that activation of previously activated cells was sufficient to reduce approach behavior in a paced mating paradigm and receiving intromissions in a home cage mating paradigm. An inhibition approach using DREADD would have been a great complement to this study as it would have examined if activation of the cells was required. Moreover, additional tests for sexual motivation would have greatly strengthened the overall conclusions.*
>
> ***Reviewer #3 (Public Review):***
>
> *[…] Weaknesses:*
>
> *(1) Their activity-dependent labeling strategy is not exclusive to mating completion but instead includes all neurons active before, during, and after the social encounter. In the manuscript, the authors did not discuss the time course of Fos activation or the*

> *timeframe of the FosTRAP labeling strategy. Fos continues to be expressed and is detectable for hours following neural activation. Therefore, the FosTRAP strategy also labels neurons that were activated 3 hours before the injection of 4-OHT. The original FosTRAP2 paper which is cited in this manuscript (DeNardo et al, 2019) performed a detailed analysis of the labeling window in Supplementary Figure 2 of that paper. Here is quoted text from that paper: "Resultant patterns of tdTomato expression revealed that the majority of TRAPing occurred within a 6-hour window centered around the 4-OHT injection." Thus, the FosTRAP "mating completion" groups throughout this manuscript also include neurons activated 3 hours before mating completion, which includes neurons activated during appetitive and consummatory mating behaviors.*
>
> *This makes all of the FosTRAP data very difficult to interpret. Compounding this is the issue that the two groups the authors compare in their experiments are females administered 4-OHT following appetitive investigation behaviors (with the male removed before mating behaviors occurred) and females administered 4-OHT following mating completion. The "appetitive" group labeled neurons activated only during appetitive investigation, but the "completion" group labeled neurons activated during appetitive investigations, consummatory mating bouts, and mating completion. Therefore, in the brain-wide analysis of Figure 2, it is impossible to identify brain regions that were activated exclusively by mating completion and not by consummatory mating behaviors. This could have been achieved if the "completion" group was compared to a group of females that had commenced consummatory mating behaviors but were separated from the male before mating was completed. Then, any neurons labeled by the "completion" FosTRAP but not the "consummatory" FosTRAP would be neurons specifically activated by mating completion. In the current brain-wide analysis experiments, neurons activated by consummatory behaviors and mating completion can not be disassociated.*
>
> *This same issue is present in the interpretation of the chemogenetic activation data in Figure 6. In the experiments of Figure 6, the authors are activating neurons naturally activated during consummatory mating behaviors as well as those activated during mating completion.*

We appreciate the reviewers comments and concerns about the TRAP method.

First, we agree that the FosTRAP method does not have the sensitivity to separate ensembles that happen within a short time window. From our preliminary results, we have observed that the cells that inject 4-OHT after mating completion induce more tdTomato cells in the MPN than injection after appetitive behavior or consummatory behavior (Author response image 1).

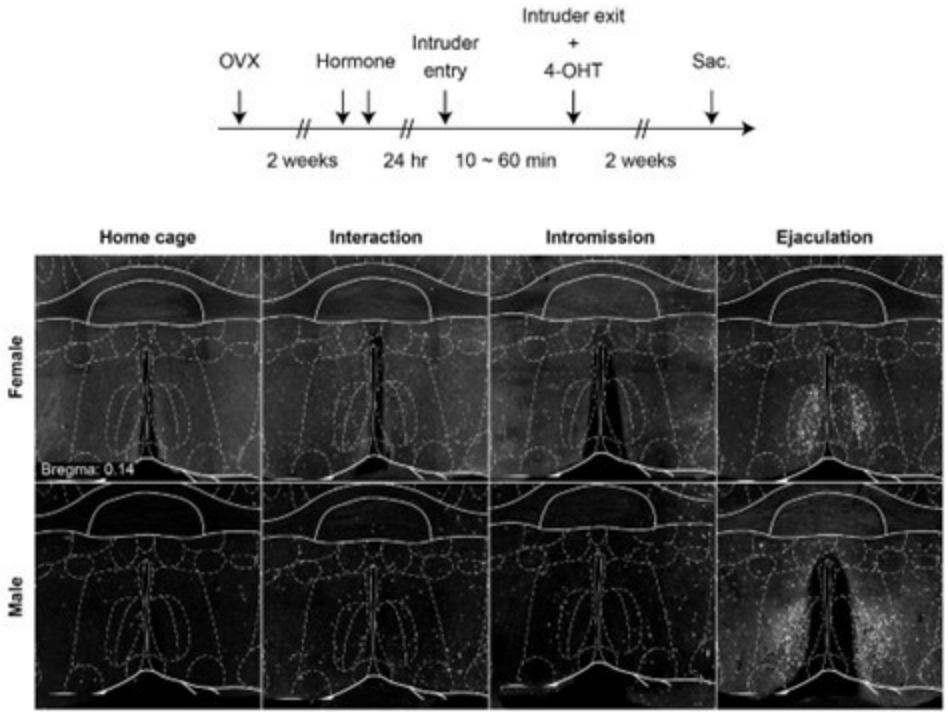
To further compare the difference between the “consummatory” and “completion” ensemble, we included an additional cohort where we TRAP cells responding to consummatory behavior. This cohort is added to Figure 2, 6, S3, S4, S9, S10 and S11. From the whole brain mapping of TRAP cells, we found that many hypothalamic and extended amygdala areas including the medial preoptic area, and the bed nucleus of stria terminalis were shown to have significantly larger tdTomato+ cell density in the completion group than in the appetitive group while there was a tendency that the consummatory group also had larger cell density than the appetitive group. In the Gq-DREADD experiment, we found that the Completion-hM3Dq group but not the Consummatory-hM3Dq group showed the reduction of sexual motivation of the female mouse in the self-paced mating assay (Figure 6). The Completion-hM3Dq group but not the Consummatory-hM3Dq group also showed significantly low intromission events and tended to show lower receptivity in the home cage mating assay (Figure S10). Furthermore, post-hoc histological analysis also showed that the c-Fos+ and TRAP labeled cells in the MPN tended to be the larger in the Completion-hM3Dq group than in the Consummatory-hM3Dq group (Figure S9). These results, together with the *in vivo* Calcium

imaging experiments in Figure 3, 4 and 5, suggests that the MPN contains male-ejaculation responsive cells that are distinct with the male-mounting responsive cells and that they are sufficient to suppress female sexual motivation.

However, it is true that with the current state of mouse genetic tools, we do not have any methods with higher time accuracy. We have discussed the limitations of FosTRAP method regarding its low time sensitivity in the Discussion section.

Author response image 1.

Representative image showing TRAP labeling in the MPN after mating completion and intromission



> *(2) This study does not definitively show that the female mice used in this study display decreased sexual motivation after the completion of mating. The females exhibit reduced interaction with males that had also just completed mating, but it is unclear if the females would continue to show reduced interaction time if given the choice to interact with a male that was not in the post-ejaculatory refractory period. Perhaps, these females have a natural preference to interact more with sexually motivated males compared to recently mated (not sexually motivated) males. To definitively show that these females exhibit decreased sexual motivation the authors should perform two control experiments: 1) provide the females with access to a fully sexually motivated male after the females have completed mating with a different male to see if interaction time changes, and 2) compare interaction time toward mated and non-mated males using the self-paced mating assay. These controls would show that the reduction in the interaction time is because the females have reduced sexual motivation and not because these females just naturally interact with sexually motivated males more than males in the post-ejaculatory refractory period.*

We highly appreciate the reviewers comments regarding the interpretation of the self-paced mating assay. To address the concerns, we added an experiment where the female subjects were introduced to a novel sexually motivated male mice in the self-paced mating assay

immediately after receiving ejaculation (Figure S2). As result, we found that similar to the self-paced mating assay using the same male animal, the female subject spends significantly more time in the isolation zone on the post-ejaculation day when compared to the pre-ejaculation day.

> *(3) It is unclear how the transient 90-second response of these MPOA neurons following the completion of mating causes the prolonged reduction in female sexual motivation that is at the minutes to hours timeframe. No molecular or cellular mechanism is discussed.*
>
> *(4) The authors discuss potential cell types and neural population markers within the MPOA and go into some detail in Figure S3. However, their experiments are performed with only the larger excitatory and inhibitory MPOA neural populations.*

While the molecular or cellular mechanism of prolonged activity of MPOA neurons is critical to understand the neural mechanism of how sustained neural activity in the MPOA suppress female sexual motivation, it is out of the reach of the current manuscript and a subject of future studies. We have added a section in the discussion part to further discuss the potential molecular mechanisms.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *If the authors haven't already, it would be useful if the authors could make the brain-wide analysis of Fos activity publicly available.*

We have distributed the data to https://dandiarchive.org/

> *I would also make sure the n's are included in each Figure Legend for each panel (some are missing in the Supplementals).*

We appreciate the comment, we have added the number of subjects to Figure 3, 4, 5.

> *It would also be best to provide clearer labels to some of the Figures, for example, Figure 5D, the Types should also be labeled with what behaviors they correspond to.*

We appreciate the comment. Figure 5 is focused on post-ejaculation neural activity. The cell types are categorized based neural activity after experiencing male ejaculation, it does not correspond to any behaviors.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) A first recommendation is to replace the use of the term "mating completion" with "ejaculation". Male and female rodents display a period of reduced approach behavior following display or experiencing ejaculation, which is referred to as the post-ejaculatory interval. The current studies investigate the neural ensemble that contributes to this post-ejaculatory interval in female mice. In addition, male and female rodents will display a prolonged period of sexual inactivity referred to as satiety, which is typically observed after repeated display or experience of ejaculations. The current studies do not investigate satiety. Moreover, in the current studies, female mice appeared to display approach behavior (time in the interaction zone) even within the 10 minutes following experiencing ejaculation (Fig 1F). Hence, the term "completion" is not accurate and should be replaced by "ejaculation" in all figures and throughout the manuscript. Replacing completion with ejaculation will also clarify what defines "onset of*

> *completion", which this reviewer assumes refers to the onset of ejaculatory behavior observed in the male.*

Thank you for the comment. We agree that the mating completion was inappropriate. We have changed the wording to ejaculation or post-ejaculatory period.

> *(2) Likewise, a variety of other terms and descriptions need to be adjusted for consistency and accuracy. For example, "room" when referring to the interaction or isolation zones; "onset of mating completion" when referring to ejaculation; "male intruder" to refer to the introduction of the male mating partner, but using a term typically used for an intruder-resident aggression test. Replacing these terms will aid in reducing confusion for the reader and more accurately describe the behavioral parameters.*

We appreciate the comment. We have updated the terms “male intruder” to “partner”, “room” to “area” or “zone”.

> *(3) The use of the paced mating paradigm is a strength of these studies. This paradigm has been widely used and validated to study female sexual behavior in rodents. Please refer to recent reviews and landmark papers using this paradigm in addition to the current cited papers to better reflect the vast wealth of studies that previously reported the behavioral data that were replicated in this study.*

We have added a section discussing the self-paced mating assay, its merits and caveats P8.

> *(4) In the paced mating test, females can pace the receipt of sexual stimulation, and latencies to withdraw and return to the male-containing chamber are considered indicators of sexual motivation. Female withdrawal will increase with the intensity of the sexual stimulation and latency to return is longer following ejaculation. Paced mating is thus a balance of approach and withdrawal behaviors that increases reward and likelihood of pregnancy for females. Moreover, ejaculation-induced withdrawal and longer latencies to return and approach are altered by hormonal status and by the introduction of a novel male partner. Thus, female sexual behavior is complex and withdrawal behavior (in this paper measured as time spent in an isolation zone) needs to be interpreted with caution and not simply referred to as sexual motivation. I recommend expanding the description of the paradigm to highlight the strengths and limitations of this paradigm and use caution to interpret time spent in the isolation zone as a lack of sexual motivation. I also recommend referring to the period after ejaculation as the post-ejaculatory interval (instead of completion).*

Thank you for the comment. We have changed the wording in the manuscript to adjust the way it refers to sexual motivation.

> *(5) In the current paper, time in the isolation zone and the number of transitions are used as the behavioral measures. Latencies, which are typically included in paced mating studies, were missing from the data. If data are available for latencies to withdraw and return to the interaction zone after mount, intromission, and ejaculation, please add these data. If such data were not collected or are not available, please recognize this caveat.*

Thank you for the comment. In figure 1, which all animals did experience male ejaculation, we added latency analysis (Figure 1I and 1P). The result indicates as suggested in the literature, female mice took significantly longer to return the interaction zone after male-ejaculation.

> *(6) The brain-wide mapping study of cFos expression after ejaculation confirms and extends prior findings, mostly in rats. Please reference prior papers in female rodents showing cFos after ejaculation and discuss how the current data replicate or differ from prior data.*

In the manuscript P8 L351, we have referred to Pfaus et al., 1993 to discuss the similarity in the c-Fos expression pattern studied in rats. We have further added descriptions to emphasize the similarity between the two datasets.

> *(7) A paragraph describing the specific cell types that are activated in the MPOA is an essential part of the study and is described in detail, but only shown in supplementary figures. Given the emphasis on this particular part of the study, a recommendation is to incorporate these data as a regular figure instead of supplementary material.*

While we greatly appreciate the comment, we consider that the molecular characterization of MPOA neurons are not the main focus of the paper and decided to keep it in the supplementary figure.

> *(8) Calcium imaging studies were performed in the home cage for obvious practical reasons. However, in the home cage testing, the females withdraw from the males using a different approach and do not exit an interaction zone through a division. There may also be differences in the male sexual behavior patterns and thus the stimulation that females receive from the male. Yet, it appears that ejaculation induces similar patterns of neural activation in this paradigm. Thus, it is likely that neuron activation is a result of receiving ejaculation, rather than withdraw behavior. Please briefly discuss the comparisons between the cFos and calcium imaging conclusions in these two different paradigms.*

We have added a section discussing the self-paced mating assay, its merits and caveats P8. Withdrawal and latency and its interpretation is discussed in this section.

> *(9) The final study includes the manipulation of ejaculation-activated neurons in the MPOA using DREADD. This study was limited to show that activation of previously activated cells was sufficient to reduce approach behavior in a paced mating paradigm and receiving intromissions in a home cage mating paradigm. An inhibition approach using DREADD would have been a great complement to this study as it would have shown if activation of the cells was required. Moreover, additional tests for sexual motivation, such as partner preference tests would have greatly strengthened the results since a lack of entering an interaction zone can also be explained by impaired sensory processing or locomotor behavior. Finally, CNO also appeared to impact time in the isolation zone for a subset of animals in the ejaculation (completion) control group and the appetitive group. These effects didn't reach statistical significance, but groups also had low sample sizes (n=6-7) and may thus have been underpowered. The recommendation is to include these caveats and shortcomings in the discussion of these results.*

We appreciate the comments. We first added an inhibitory approach to show the necessity of MPOA neurons. As result, we found that the inhibition of these neurons did not affect the behavior in the self-paced mating assay but increased the subjects sexual receptivity (Figure S11). For the low sample size, we have added a power analysis in the statistical section.

> *(10) The studies utilized ovariectomized females with hormone priming. Since sexual receptivity in females is highly dependent on the hormonal milieu, the authors are*

> *encouraged to add an explanation of why ovariectomized females were used and if the results may have differed in cycling females.*

We appreciate the comments. The female subjects used in the TRAP experiment will be needing to experience ejaculation from the male mice twice, once to label the cells, and second during the reactivation. In order to avoid pregnancy during the first experience, we ovariectomized the female and controlled their hormonal conditions. This method has been used successfully in other sexual behavior studies (Yang et al., 2013, Ring., 1944.). This was described in P11. We have further demonstrated in Figure 1N-T that female mice were not ovariectomized and were under the natural estrus cycle showed similar suppression of sexual interaction after the completion of mating. The manuscript was updated to discuss that the behavior change after mating completion is not dependent on the ovary.

> *(11) Overall, the paper lacks references to relevant prior studies. For example, many studies have been reported over the past 2-3 decades about the effects of female rodent sexual behavior on activation in the brain and the effects of different vaginocervical stimulation on pregnancy and fertility. It is absolutely the case that much remains unknown about the complex neural circuitries that control behavior during the post-ejaculatory interval and sexual satiety in both male and female rodents, but studies have indicated roles for hypothalamic areas, bed nucleus of the stria terminals, ventral tegmental area, posterior thalamus, and prefrontal cortex. Hence, the current introduction and discussion do not adequately summarize or acknowledge these prior investigations and therefore place these new findings in the context of what was previously known.*

We appreciate the comment and added references to P2 L65, P8 L355-357 to discuss existing literature about c-Fos mapping analysis after ejaculation or genital stimulation in female rats.

> *(12) Finally, sample sizes appear to be modest, ranging n=4-8 (except n=14 in the completion group in Figure S7) and vary between groups within and between studies. Please explain in the methods section how sample sizes were pre-determined and acknowledge if studies may have potentially been underpowered.*

The sample size for behavior experiments in this study were n = 6-9. This was predetermined based on previous studies examining female sexual behavior (Ishii et al. 2017, Liu et al. 2022, Yin et al. 2022). To further examine the number of animals required for our behavioral experiments, we pooled data used in this study and conducted a power analysis (n = 111 pooled data, control n = 94, stim n = 17). We conducted a power analysis using the variance calculated from pooled average time in isolation zone. These data were pooled from control animals in each experiment (eg. animals with GFP control virus injected, saline injected, etc.). The average time in isolation zone in the after ejaculation or after reactivating the completion cells was 420 ± 210 seconds, and 49 ± 91 seconds in the control group (mean ± s.d.). Within this population, we found that 5 animals were sufficient to detect the difference ($p < 0.05$, power = 0.8) in Students t-test. We have added this explanation in the supplemental experimental procedure, page P18, line 817-827.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *The authors should discuss the fact that the FosTRAP2 strategy labels neurons activated 3 hours before the 4-OHT injection. As the manuscript is written, it seems to suggest that the 4-OHT injection given following mating completion only labeled neurons activated during mating completion. This is very misleading. I respect the amount of work and rigor that went into these experiments. The single-cell imaging, implementation of the FosTRAP strategy, and behavioral analysis are all well executed. Novel insights into the*

> *neural regulation of female sexual drive can be gleaned from the neural imaging experiments. Unfortunately, the limitations of the FosTRAP strategy make those studies very difficult to interpret, and therefore, a more candid discussion and re-interpretation of the data from the FosTRAP experiments is needed.*

We appreciate the reviewers comments and concerns about the TRAP method.

First, we agree that the FosTRAP method does not have the sensitivity to separate ensembles that happen within a short time window. From our preliminary results, we have observed that the cells that inject 4-OHT after mating completion induce more tdTomato cells in the MPN than injection after appetitive behavior or consummatory behavior (Author response image 1).

To further compare the difference between the “consummatory” and “completion” ensemble, we included an additional cohort where we TRAP cells responding to consummatory behavior. This cohort is added to Figure 2, 6, S3, S4, S9, S10 and S11. From the whole brain mapping of TRAP cells, we found that many hypothalamic and extended amygdala areas including the medial preoptic area, and the bed nucleus of stria terminalis were shown to have significantly larger tdTomato+ cell density in the completion group than in the appetitive group while there was a tendency that the consummatory group also had larger cell density than the appetitive group. In the Gq-DREADD experiment, we found that the Completion-hM3Dq group but not the Consummatory-hM3Dq group showed the reduction of sexual motivation of the female mouse in the self-paced mating assay (Figure 6). The Completion-hM3Dq group but not the Consummatory-hM3Dq group also showed significantly low intromission events and tended to show lower receptivity in the home cage mating assay (Figure S10). Furthermore, post-hoc histological analysis also showed that the c-Fos+ and TRAP labeled cells in the MPN tended to be the larger in the Completion-hM3Dq group than in the Consummatory-hM3Dq group (Figure S9). These results, together with the *in vivo* Calcium imaging experiments in Figure 3, 4 and 5, suggests that the MPN contains male-ejaculation responsive cells that are distinct with the male-mounting responsive cells and that they are sufficient to suppress female sexual motivation.

However, it is true that with the current state of mouse genetic tools, we do not have any methods with higher time accuracy. We have discussed the limitations of FosTRAP method regarding its low time sensitivity in the Discussion section.

> ***Editor notes:***
>
> *Should you choose to revise your manuscript, please include full statistical reporting in the main text including test statistic, degrees of freedom, an exact P value.*

Thank you for the comment. The statistical values were added to the manuscript.

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