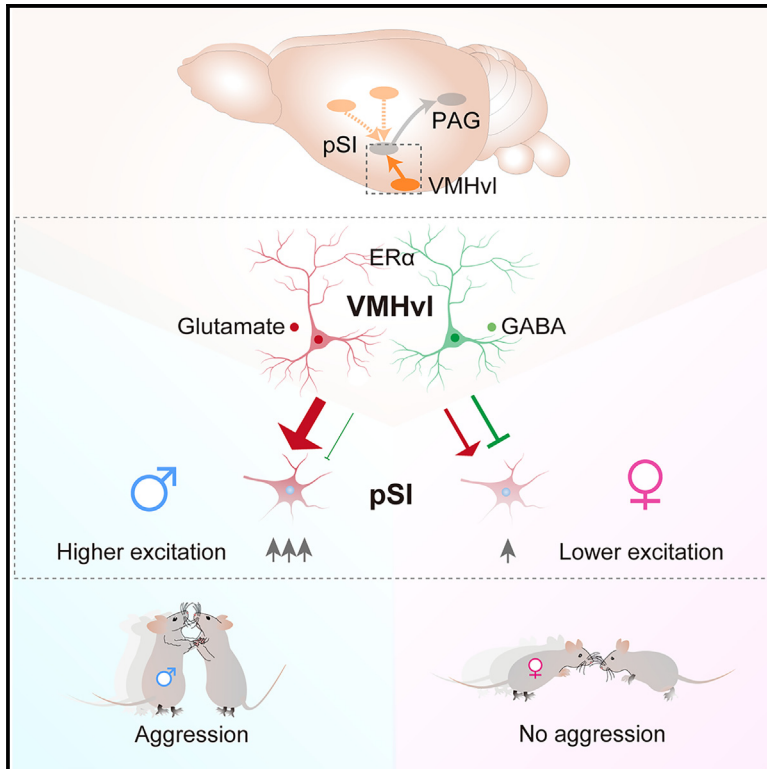


A hypothalamic-amygdala circuit underlying sexually dimorphic aggression

Graphical abstract



Authors

Zhenggang Zhu, Lu Miao, Kaiyuan Li, ..., Li Sun, Yan-qin Yu, Shumin Duan

Correspondence

yanqinyu@zju.edu.cn (Y.-q.Y.), duanshumin@zju.edu.cn (S.D.)

In brief

By identifying a hypothalamic-amygdala circuit that mediates male-biased aggression, Zhu et al. demonstrated that dominant excitatory projections within the VMHvl-pSI circuit in male mice lead to increased pSI activity and heightened aggression, revealing a sexually distinct excitation-inhibition balance during social behaviors.

Highlights

- The VMHvl-pSI circuit mediates male-biased aggression
- Males exhibit stronger excitatory VMHvl-pSI connections
- Females show stronger inhibitory VMHvl-pSI connections
- Heightened hypothalamic input boosts pSI activity, increasing male aggression

Zhu et al., 2024, *Neuron* 112, 1–16

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<https://doi.org/10.1016/j.neuron.2024.06.022>

Article

A hypothalamic-amygdala circuit underlying sexually dimorphic aggression

Zhenggang Zhu,^{1,2,4,9} Lu Miao,^{1,2,4,9} Kaiyuan Li,^{1,2,4} Qingqing Ma,^{1,2,4} Lina Pan,^{1,2,4} Chenjie Shen,^{1,2,4} Qianqian Ge,^{1,2,4} Yonglan Du,^{1,2,4} Luping Yin,⁶ Hongbin Yang,^{1,2,4} Xiaohong Xu,⁵ Ling-Hui Zeng,⁷ Yijun Liu,^{1,2,4} Han Xu,¹ Xiao-ming Li,^{1,3,4} Li Sun,^{1,2,4} Yan-qin Yu,^{1,2,3,4,7,*} and Shumin Duan^{1,2,4,7,8,10,*}

¹Department of Neurology of Second Affiliated Hospital and School of Brain Science and Brain Medicine, Zhejiang University School of Medicine, Hangzhou 310058, China

²Liangzhu Laboratory, MOE Frontier Science Center for Brain Science & Brain-Machine Integration, State Key Laboratory of Brain-Machine Intelligence, Zhejiang University, Hangzhou 311121, China

³Nanhu Brain-Computer Interface Institute, Hangzhou 311100, China

⁴NHC and CAMS Key Laboratory of Medical Neurobiology, Zhejiang University, Hangzhou 310058, China

⁵Institute of Neuroscience and Key Laboratory of Primate Neurobiology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

⁶Westlake Laboratory of Life Sciences and Biomedicine, Institute of Biology, School of Life Sciences, Westlake Institute for Advanced Study, Westlake University, Hangzhou 310024, China

⁷Key Laboratory of Novel Targets and Drug Study for Neural Repair of Zhejiang Province, School of Medicine, Hangzhou City University, Hangzhou 310015, China

⁸Research Units for Emotion and Emotion Disorders, Chinese Academy of Medical Sciences, Hangzhou, China

⁹These authors contributed equally

¹⁰Lead contact

*Correspondence: yanqinyu@zju.edu.cn (Y.-q.Y.), duanshumin@zju.edu.cn (S.D.)

<https://doi.org/10.1016/j.neuron.2024.06.022>

SUMMARY

Male animals often display higher levels of aggression than females. However, the neural circuitry mechanisms underlying this sexually dimorphic aggression remain elusive. Here, we identify a hypothalamic-amygdala circuit that mediates male-biased aggression in mice. Specifically, the ventrolateral part of the ventromedial hypothalamus (VMHvl), a sexually dimorphic region associated with eliciting male-biased aggression, projects densely to the posterior substantia innominata (pSI), an area that promotes similar levels of attack in both sexes of mice. Although the VMHvl innervates the pSI unidirectionally through both excitatory and inhibitory connections, it is the excitatory VMHvl-pSI projections that are strengthened in males to promote aggression, whereas the inhibitory connections that reduce aggressive behavior are strengthened in females. Consequently, the convergent hypothalamic input onto the pSI leads to heightened pSI activity in males, resulting in male-biased aggression. Our findings reveal a sexually distinct excitation-inhibition balance of a hypothalamic-amygdala circuit that underlies sexually dimorphic aggression.

INTRODUCTION

Animals have evolved various biological variables to maximize their survival and reproduction. One of the most apparent biological factors is sex, often enabling male or female animals to behave differently in their competition for mates or resources.^{1,2} In humans, it has been reported that men show higher aggression than women.^{3,4} Similarly, male animals, such as rodents, show abundant aggression beyond the juvenile stage, whereas laboratory female rodents have a much lower tendency to attack in adulthood.^{5–8} These quantitatively distinct aggressive behaviors of the sexes gradually form during development and become more stable in adulthood.^{5,9} Predictably, the associated sexually differentiated neural circuits for social behaviors are similarly

observed as shaped during development and stabilized upon adulthood.^{1,7,10–12} Since aggressive attacks are executed by brain circuits, it is presumed that these sexually differentiated neural circuits drive sexually dimorphic aggression in adulthood. However, how the neural pathways that drive male-biased aggressive behavior but neglect to trigger female aggression in adulthood have remained elusive.

Despite the discovery of some sex-specific brain nodes for aggression in invertebrates (such as *Drosophila*),^{13–16} aggression-related brain regions in mammals are mostly shared between the two sexes.^{9,17,18} Silencing aggression-relevant areas similarly suppresses aggressive behaviors in both male and female mice^{8,9,17} but their efficiency in eliciting aggression could vary according to different genetic backgrounds or reproductive

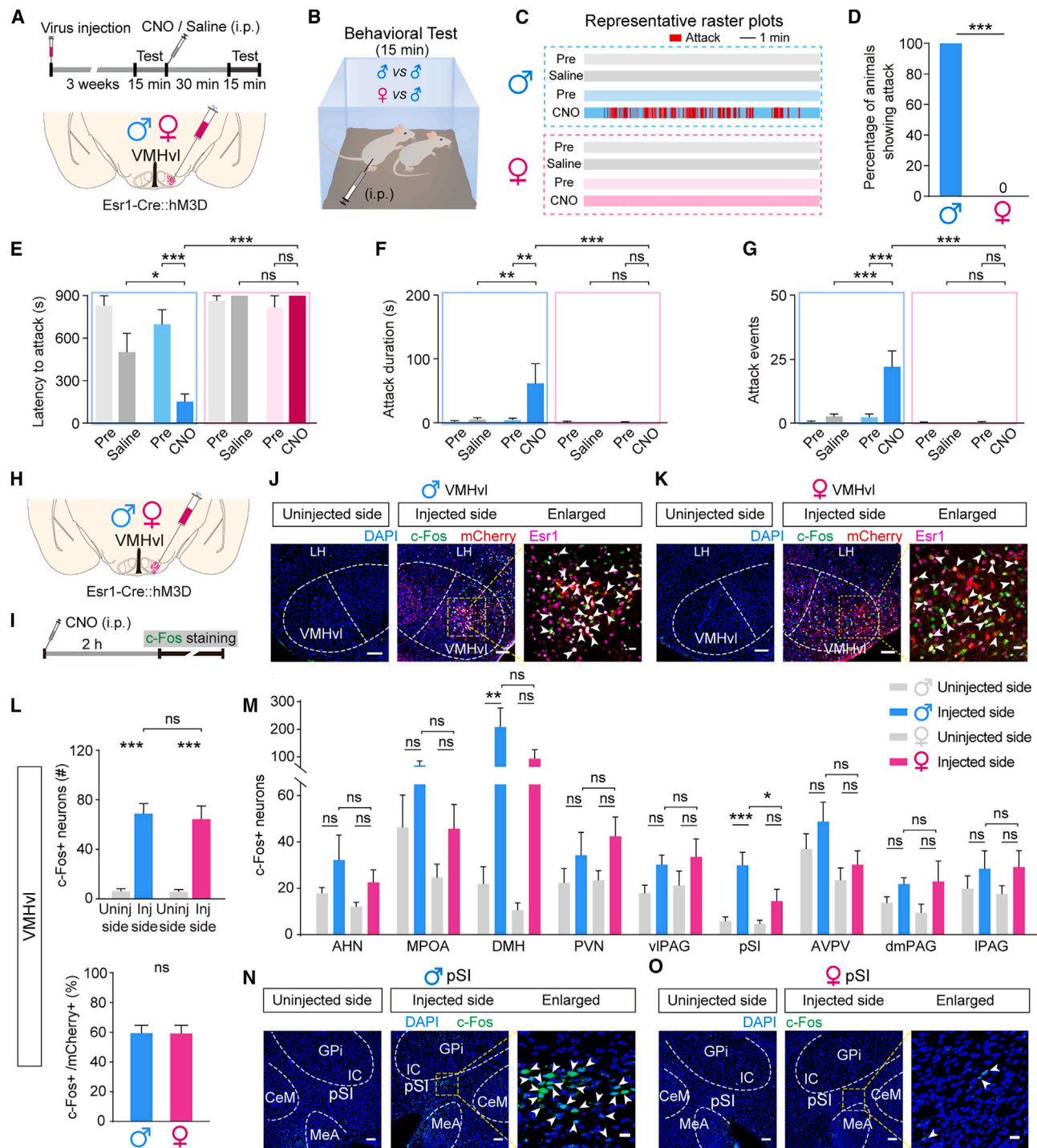


Figure 1. Pharmacogenetic activation of VMHvl^{Esr1} neurons induces male-specific aggression and differentially activates downstream brain regions in both males and females

(A and B) Strategy of pharmacogenetically activating VMHvl^{Esr1} neurons (A) and behavioral paradigm (B) in male and female mice.

(C) Raster plots of attack behavior recorded during 15-min encounters in the saline control (day 1) and CNO groups (day 2) from male (upper) and female (lower) mice.

(D–G) Percentage of attack trials (D), latency to the first attack (E), attack duration (F), and attack events (G), before or after saline or CNO injection in male and female mice.

(H and I) The strategy of examining the c-Fos expression (H) and timeline (I) during pharmacogenetic activation of the VMHvl^{Esr1} neurons when a mouse was solitary in its home cage.

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stages of two sexes.^{8,19–24} For example, the estrogen receptor- α (Esr1)-positive or progesterone receptor (PR)-positive ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) neurons promote robust male aggression but elicit less potent aggression in sexually inexperienced females.^{6,8,22,25–27} The sexually differential properties of these aggression-eliciting neurons contributing to sexually dimorphic aggression remain understudied.^{1,9}

In contrast to these sexually dimorphic aggression-eliciting modules, certain brain areas induce female aggression by overriding the requirement for sex-specific conditions. The posterior substantia innominata (pSI) notably overrides the female-related tendency to elicit comparable aspects to male aggression through its projection to the periaqueductal gray (PAG),²⁸ a premotor region for upstream aggression-related areas (such as the VMHvl).^{28,29} As the pSI elicits attacks in both males and females, it could therefore serve as a sexually convergent region of focus for research aiming to illuminate the underlying mechanisms of sexually dimorphic aggression. However, the related interactions between brain modules that commonly elicit aggression in both sexes and those that elicit sexually dimorphic aggression remain understudied. Our study demonstrates that the sexually deferential glutamatergic and GABAergic connection strength between VMHvl^{Esr1} and pSI neurons plays a pivotal role in modulating sexually dimorphic aggression.

RESULTS

Sexually dimorphic activation of neurons downstream of the VMHvl

To understand how the neural circuits are involved in sexually dimorphic aggressive behaviors, we focused on the VMHvl, a social node that drives robust aggressive behaviors in mice.^{8,22,30} We first aimed to validate the role of VMHvl in male and female aggression, particularly as such aggression responses, especially those of Esr1/PR-positive neurons, are known to be complicated by differences across various genetic backgrounds and reproductive states.^{8,9,22} We therefore pharmacogenetically activated VMHvl^{Esr1} neurons by unilaterally injecting an adeno-associated virus (AAV)-double-inverted orientation (DIO)-hM3Dq-mCherry virus into the VMHvl of Esr1-Cre C57BL/6 (C57) mice of both sexes (Figures 1A and S1M–S1N), where the VMHvl^{Esr1} neurons are thereby targeted by the engineered Cre-dependent designer receptors exclusively activated by designer drugs (DREADDq).³¹ Intraperitoneal (i.p.) injection of the cognate ligand clozapine-N-oxide (CNO) significantly increased aggressive behaviors in male mice. How-

ever, no observable aggression was evoked in female mice (Figures 1B–1G).

We then reasoned the activation level of VMHvl and/or its downstream brain regions³² might differ and thereby elicit disparate levels of aggression in the two sexes. We sought to test activation levels by checking the expression of the immediate early response gene c-Fos either in or downstream of VMHvl when pharmacogenetically activating VMHvl^{Esr1} neurons in male and female mice (Figures 1H and 1I). Interestingly, we observed a similar proportion of activated VMHvl^{Esr1} neurons expressing c-Fos in males and females (Figures 1J–1L), suggesting VMHvl^{Esr1} neurons to have been similarly activated in both sexes. As VMHvl^{Esr1} neurons are connected to many brain regions, we therefore then searched for related circuits by assessing the c-Fos expression of the most densely innervated major downstream targets of VMHvl^{32,33} (Figures 1M–1O and S1A–S1J). While most regions exhibited high levels of c-Fos in both sexes, the pSI was the most significantly male-biased region following pharmacogenetic activation of VMHvl^{Esr1} neurons (Figures 1M–1O, S1K, and S1L). Notably, the pSI is a newly identified area in the extended amygdala suggested to elicit similar levels of male and female aggression.²⁸

A hierarchically organized circuit between VMHvl and pSI mediates male aggression

While previous studies have reported that many labeled fibers from the VMHvl are sent to the pSI,^{32–34} it was unclear if they were fibers of passage in the pSI. We, therefore, considered if the circuit of the sexually dimorphic aggression-eliciting neurons in the VMHvl may innervate the sexually common aggression-eliciting neurons in the pSI. To label projection termini of VMHvl neurons in the pSI, a Cre-dependent, virally encoded mRuby fused to the synaptic vesicle protein synaptophysin (Syn: mRuby) was targeted to VMHvl^{Esr1} neurons in male Esr1-Cre mice (Figure 2A). We observed dense presynaptic termini in the pSI, but few in neighboring regions (Figures 2B and 2C).

To ascertain if the pSI functions as a downstream target of the VMHvl in modulating aggression, a Cre-dependent hM3Dq virus was unilaterally injected into the VMHvl of male Esr1-Cre mice to activate the VMHvl^{Esr1} neurons. We also concurrently conducted bilaterally injections of the AAV-hSyn-hGtACR1-EGFP virus into the pSI to enable optogenetic silencing of pSI neurons (Figures 2D and S2A). Chemogenetic activation of VMHvl^{Esr1} neurons via i.p. injection of CNO significantly promoted inter-male aggression (Figure 2E). This was then blocked by the photoinhibition of pSI neurons (Figures 2F–2J). We also investigated if aggression induced by the VMHvl-pSI projection could be influenced by the pSI. Given that pSI^{Thy1} neurons are critical for aggression,²⁸ we

(J and K) Representative VMHvl histological images showing the expression of hM3Dq-mCherry (red), anti-Esr1 (magenta), and c-Fos+ neurons (green) on the uninjected (left) and injected side (middle) in male (J) and female (K) mice. Scale bars, 100 μ m. Right, magnification of the boxed area; arrowheads, Esr1/c-Fos+ neurons; scale bars, 20 μ m. LH, lateral hypothalamus.

(L) c-Fos+ neurons (upper) and percentages of mCherry+ neurons expressing c-Fos (lower) on the VMHvl in male and female mice.

(M) C-Fos expression in 9 downstream targets of VMHvl^{Esr1} neurons after chemogenetic activation of the VMHvl^{Esr1} neurons in Esr1-Cre mice of both sexes.

(N and O) Representative histological image of the pSI showing the expression of c-Fos+ neurons (green) on the uninjected side (left) and the injected side (middle) in male (N) and female (O) mice. Scale bars, 100 μ m. Right, magnification of the boxed area; scale bars, 20 μ m. CeM, central amygdala, medial part; MeA, medial amygdaloid nucleus; GPI, globus pallidus internus; IC, internal capsule.

See also Figure S1.

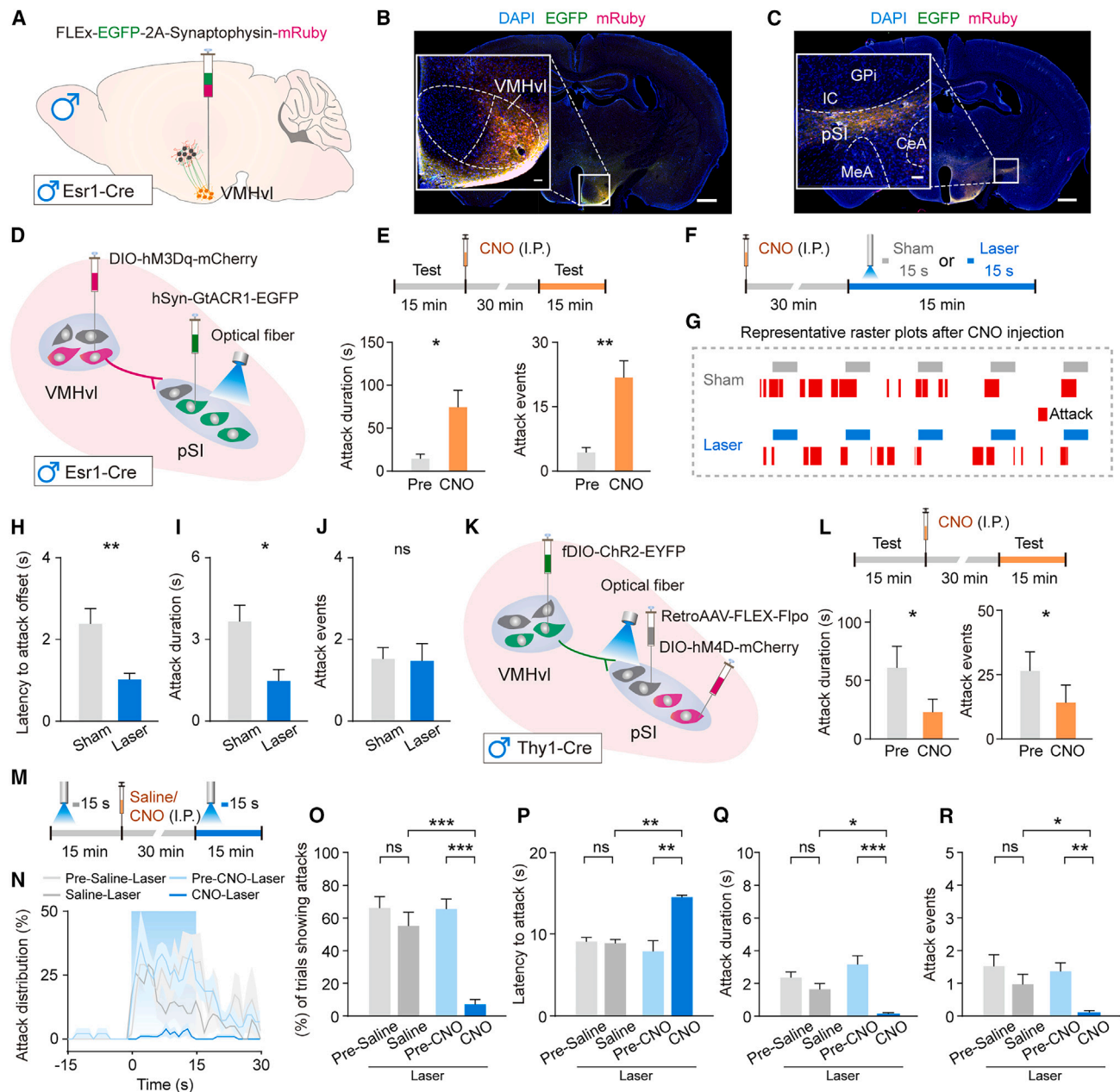


Figure 2. Hierarchically organized circuit between VMHvl and pSI mediates aggression in males

(A) Anterograde tracing of VMHvl^{Esr1} neurons in males.
 (B and C) Representative EGFP (green) and synaptophysin-mRuby (red) expression in the VMHvl (B) and pSI (C). The rectangular regions show magnified images. Scale bars, 500 μ m, and inset, 50 μ m.
 (D) Experimental design for optogenetic inhibition of pSI neurons after chemogenetic activation of VMHvl^{Esr1} neurons.
 (E) Upper, timeline for activating the VMHvl^{Esr1} neurons. Lower, attack duration and attack events before and after CNO injection.
 (F and G) Experimental timeline (F) and representative raster plots (G) of attack behavior for photo-inhibiting pSI neurons after chemogenetic activation of VMHvl^{Esr1} neurons by CNO.
 (H–J) Latency to the attack offset (H), attack duration (I), and attack events (J) when photo-inhibiting pSI neurons in sham and laser groups after CNO injection.
 (K) Experimental design for studying effects of chemogenetic inhibition of pSI^{Thy1} neurons on male aggression induced by photostimulation of the VMHvl^{Thy1} projection terminals in pSI in Thy1-Cre mice.
 (L) Upper, experimental timelines of male aggression test with chemogenetic inhibition of pSI^{Thy1} neurons. Lower, attack duration and events before and after CNO injection.

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injected Cre-dependent hM4D virus into the pSI of male Thy1-Cre mice to silence the pSI^{Thy1} neurons. Simultaneously, a Cre-dependent retrograde AAV-DIO-Flp recombinase (Flpo) virus was injected into the pSI, followed by the injection of the Flpo-dependent channelrhodopsin-2 (ChR2) virus into the VMHvl to label and optogenetically activate the VMHvl^{Thy1}-pSI projection (Figures 2K and S2B). Suppression of aggression was confirmed when chemogenetically inhibiting pSI^{Thy1} neurons in males (Figure 2L), while aggressive behaviors were enhanced upon optogenetically activating the VMHvl^{Thy1}-pSI projection (Figures 2M and 2N). However, a marked decrease in this optogenetically enhanced aggressive behavior was noted when pSI^{Thy1} neurons were chemogenetically inhibited in these mice (Figures 2O–2R). These results affirmed that VMHvl-induced male aggression is mediated via pSI neurons.

Hierarchical circuitry linking the VMHvl, pSI, and PAG in the mediation of male aggression

The PAG in the midbrain is known to facilitate the motor output of aggressive attacks.^{28,29,35} PAG projecting pSI (pSI^{PAG}) neurons, which largely overlap with pSI^{Thy1} neurons, play a critical role in gating the action outputs of aggression.²⁸ To further understand the connection between the VMHvl and pSI^{PAG} neurons, we utilized the trans-synaptic retrograde tracing of pSI^{PAG} neurons in male C57 mice (Figures 3A–3C). We confirmed dense connectivity between VMHvl and pSI^{PAG} neurons (Figure 3D). In addition, we injected both retrograde retroAAV-EYFP or Retrobeads into the PAG and AAV-calcium/calmodulin-dependent protein kinase II (CaMKII)-ChR2 virus into the VMHvl of male C57 mice. The CaMKII marker was chosen due to the involvement of excitatory neurons in the VMHvl in aggression.^{27,29} We then measured the excitatory postsynaptic currents (EPSCs) in pSI^{PAG} neurons while optogenetically activating the VMHvl^{CaMKII}-pSI projections (Figure 3E). The functional connection of VMHvl to pSI^{PAG} neurons was confirmed (Figures 3F–3H).

To determine whether aggression induced by optogenetic stimulation of pSI^{PAG} neurons is influenced by suppressing VMHvl neurons, we evaluated aggressive behaviors upon activation of the pSI^{PAG} neurons, concurrently silencing those of the VMHvl in male mice. For this, we injected both the retroAAV-Cre virus into the PAG and Cre-dependent ChR2 virus into the pSI to optogenetically activate the pSI^{PAG} neurons while simultaneously silencing VMHvl^{CaMKII} neurons using i.p. injection of the CNO in male mice injected with the AAV-CaMKII-hM4Di virus into the VMHvl (Figures 3I–3L and S2C). Aggressive behavior was largely blocked when VMHvl^{CaMKII} neurons were inhibited (Figure 3M). However, photostimulation of pSI^{PAG} neurons still elicited reliable aggressive behaviors, even when the VMHvl^{CaMKII} neurons were silenced (Figures 3N–3Q). This indicates that the VMHvl is not downstream of the pSI in mediating the expression of aggression. This circuitry of aggression elicitation is therefore suggested to be hierarchically organized from the VMHvl to the pSI, and then to the PAG.

Bidirectional regulation of male aggression by the VMHvl^{CaMKII}-pSI and VMHvl^{VGAT}-pSI projections

Since VMHvl neurons induce male-biased aggression while the pSI is effective in inducing similar aggression in both sexes,²⁸ it became important to investigate whether the VMHvl-pSI projection promotes aggression preferentially in males. We, therefore, injected the AAV-CaMKII-ChR2-mCherry virus into the VMHvl of both virgin male and female C57 mice (Figures 4A, 4B, S2F, and S2G). Approximately 89% of CaMKII-positive neurons in male mice expressed vesicular glutamate transporter 2 (VGLUT2), whereas only ~11% expressed vesicular GABA transporter (VGAT) (Figures S2D–S2E). We also confirmed excitatory synaptic inputs from VMHvl to pSI by electrophysiological recordings and validated these via the application of glutamatergic receptor antagonists (Figures 4C–4E). Interestingly, optogenetic activation of VMHvl^{CaMKII} neuronal terminals in pSI-induced aggression behavior in males but not in females (Figures 4F–4L), confirming the VMHvl^{CaMKII}-pSI projections predominantly promote aggression in males.

Single-cell RNA sequencing and *in situ* hybridization studies have also revealed inhibitory VMHvl neurons.^{8,36} However, their specific roles in aggression have remained unknown. We first investigated how the inhibitory VMHvl neurons regulate pSI neuronal activity *in vivo* by injecting viruses encoding the Cre-dependent channelrhodopsin-ChrimsonR and hSyn-GCaMP6m into the VMHvl and pSI, respectively, of male VGAT-Cre mice (Figures S2H–S2J). Decreased activity in pSI neurons was observed during fiber photometry recordings of calcium signals upon activation of the VMHvl^{VGAT}-pSI projection (Figures S2K–S2N), confirming that GABAergic VMHvl neurons suppress pSI neuronal activities in males. We further examined their role in aggression by injecting a retroAAV-FLEX-Flpo virus into the pSI and a Flpo-dependent-ChR2 virus into the VMHvl in VGAT-Cre mice (Figures 4M–4O and S3A). In this case, optically stimulating VMHvl^{VGAT}-pSI projection resulted in diminished male aggression (Figures 4P–4S; Video S1) but did not alter female aggression (Figures 4T–4W). We then assessed aggressive behaviors when suppressing the GABAergic pSI-projecting VMHvl (VMHvl^{pSI}) neurons in males and females (Figures S3B–S3J). To label upstream GABAergic VMHvl^{pSI} neurons in VGAT-Cre mice, we injected a retroAAV-FLEX-Flpo virus into the pSI and an AAV-fDIO-hM4di-mCherry virus into the VMHvl (Figures S3B and S3C). Chemogenetically inhibiting the GABAergic VMHvl^{pSI} neurons through i.p. injection of CNO enhanced inter-male aggression (Figures S3D–S3G) but failed to elicit female aggression (Figures S3H–S3J).

In summary, VMHvl^{CaMKII}-pSI and VMHvl^{VGAT}-pSI projections selectively evoke and inhibit male aggressive behaviors, respectively.

VMHvl^{Esr1}-pSI projection elicits male-biased aggression

Although only about 10% of VMHvl neurons are associated with attack behaviors, most aggression-related VMHvl neurons

(M) Schematic of the timing to test VMHvl^{Thy1}-pSI projection-triggered aggression before and after saline or CNO delivery.

(N–R) Attack distribution (N), the percentage of attack (O), latency to attack onset (P), attack duration (Q), and attack events (R) of light-induced attacks by photoactivating the VMHvl^{Thy1}-pSI projection before or after saline/CNO delivery.

See also Figure S2.

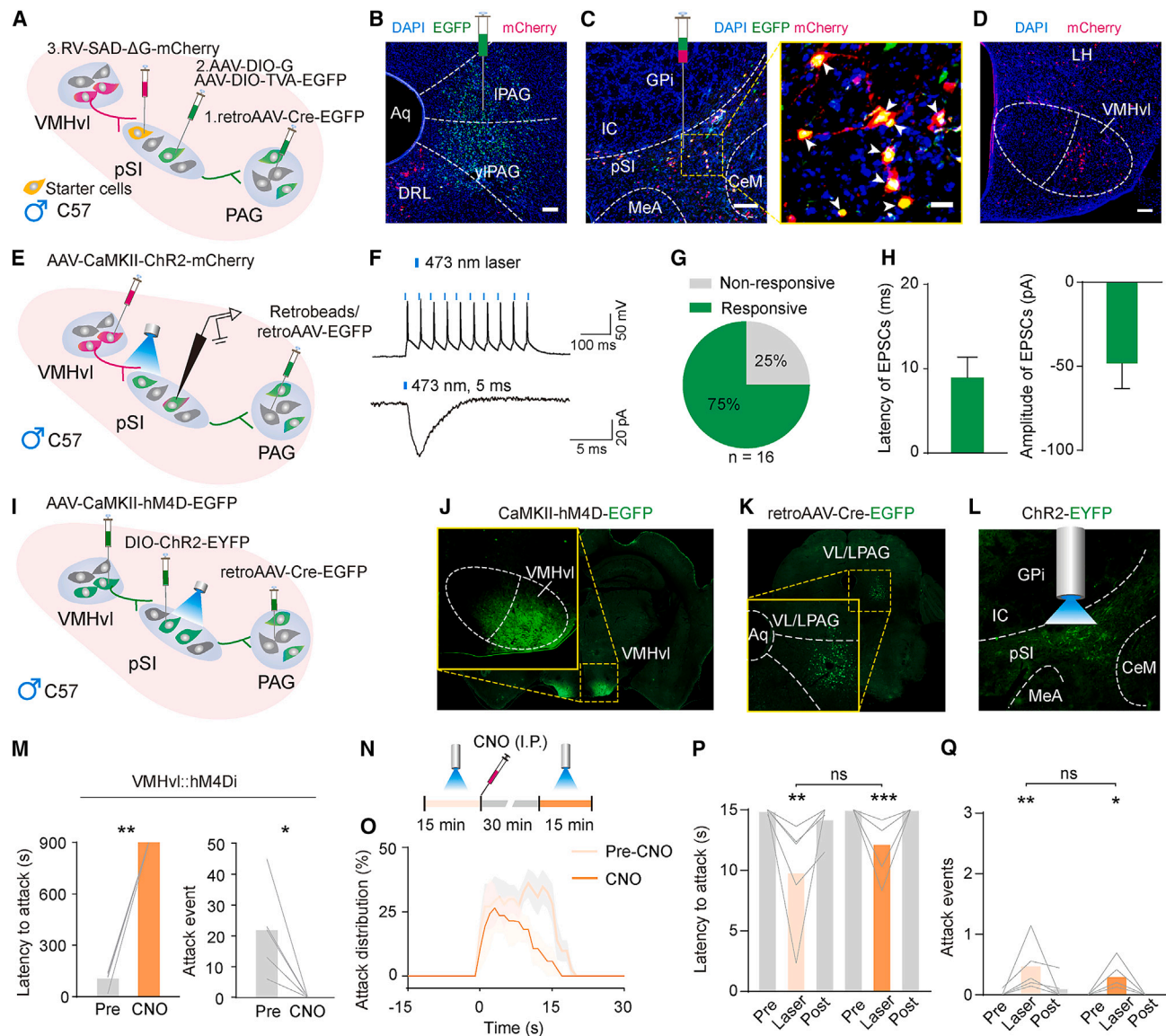


Figure 3. The VMHvl-pSI^{PAG} projection mediates aggression in males

(A) Strategy for the retrograde trans-synaptic tracing of pSI^{PAG} neurons.

(B) AAVretro-Cre-EGFP (green), and rabies virus (red) infection in the VL/LPAG. Scale bars, 100 μm. Aq, aqueduct; DRL, dorsal raphe nucleus, lateral part.

(C) Left, example image of starter neurons (yellow) in the pSI. Scale bars, 100 μm. Right, magnification of the boxed area. Arrowheads indicate the starter pSI^{PAG} neurons. Scale bars, 10 μm.

(D) Retrogradely labeled VMHvl neurons (red) from the retrograde trans-synaptic tracing of pSI^{PAG} neurons. Scale bars, 100 μm.

(E) Schematic of whole-cell electrophysiological recording from the pSI^{PAG} neurons during optogenetic activation of the VMHvl-pSI projections.

(F) Upper, representative EPSPs superposed with action potentials in a pSI^{PAG} neuron induced by photostimulation of the VMHvl-pSI projections (blue bars: light pulse at 20 Hz). Lower, light-evoked EPSC in an EGFP+ pSI neuron.

(G and H) Proportions of light-evoked responses (G), averaged latency (H, left), and amplitude (H, right) of light-evoked EPSCs were recorded in EGFP/Retrobead positive pSI neurons.

(I) Strategy for testing whether aggression induced by photoactivation of pSI^{PAG} neurons was affected by chemogenetic inhibition of VMHvl^{CaMKII} neurons.

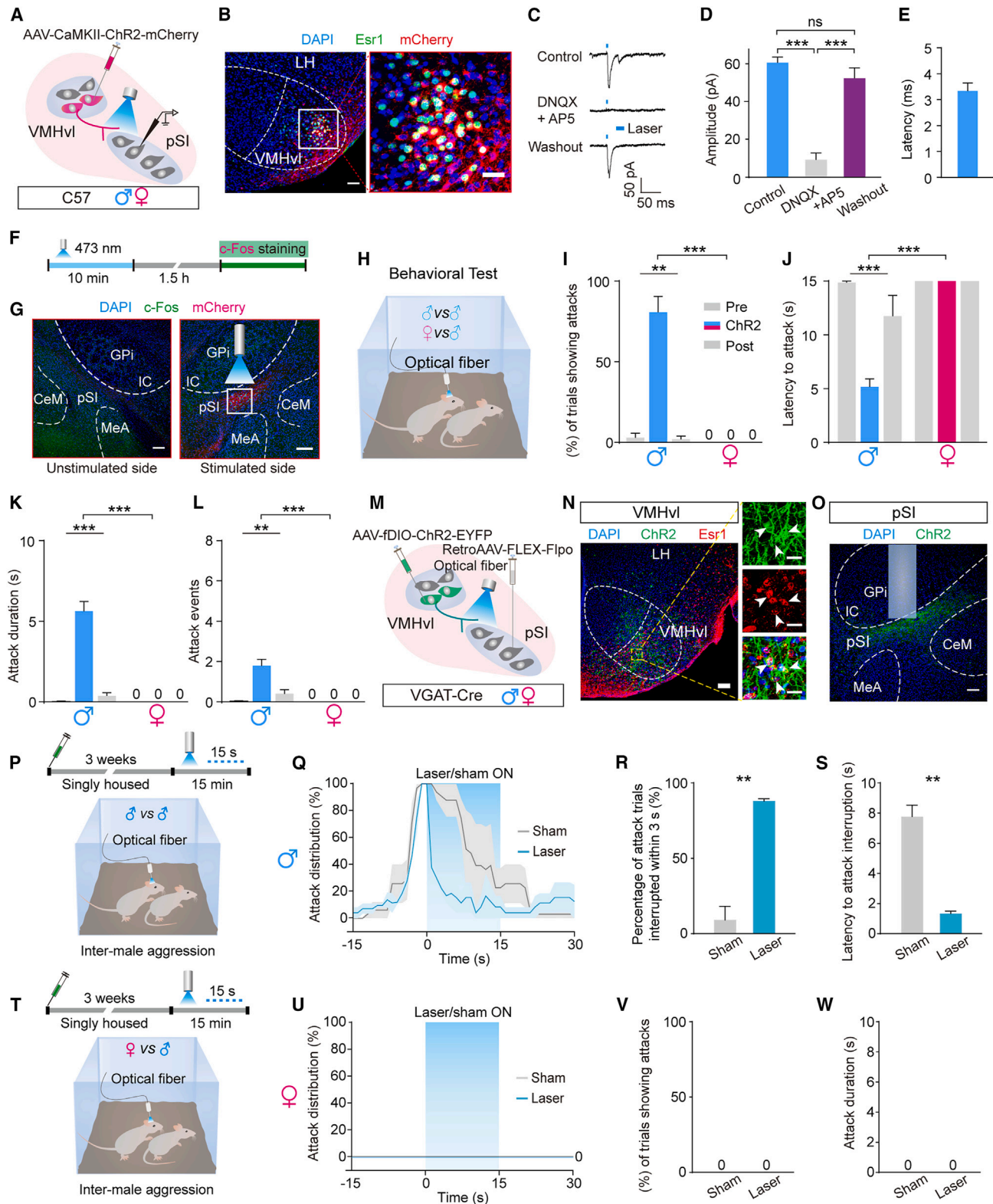
(J-L) Representative images of CaMKII-hM4D-EGFP viral infection in the VMHvl (J), retrograde AAV-retro-EGFP viral infection in the PAG (K), and DIO-ChR2-EGFP viral infection in the pSI (L). Insets, magnified images. Scale bars, 100 μm.

(M) Latency to the first attack and attack events per session of aggression before or after CNO injection.

(N and O) Schematic of male aggression test (N), and attack distribution (O), of aggression induced by photoactivation of pSI^{PAG} neurons (15 s on/30 s off) before (pre-CNO) or after (CNO) pharmacogenetic inhibition of VMHvl neurons.

(P and Q) Latency to attack onset (P) and attack events (Q) of light-induced male aggression before and after CNO delivery.

See also Figure S2.



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express *Esr1*/PR.^{8,22,30} Thus, evaluating the VMHv^{Esr1}-pSI projection in aggression is crucial for understanding the role of VMHv-pSI connections in sexually dimorphic aggression. To do this, a Cre-dependent ChR2 virus was injected into the VMHv, and subsequent aggression and mating behaviors were then assessed in male or female *Esr1*-Cre mice (Figures 5A–5D and S3K). Photostimulation of the VMHv^{Esr1}-pSI projection induced robust inter-male aggression (Figures 5E–5I; Video S2) but only elicited mild aggression behavior in female mice toward either male or female intruders (Figures 5E–5O; Video S3). No mating behaviors were observed (Figure 5E; Video S2). Since neural mechanisms link female social behaviors with estrus phases,³⁷ we then included monitoring of the estrus state of the female mice to investigate whether these behaviors induced by the photoactivation vary across estrus cycles (Figure 5P). Induced aggression by the photoactivation was similar during estrus and non-estrus phases, and notably, no significant mating behaviors were observed during these stimulations (Figures 5Q–5U).

Together, we demonstrated that the VMHv^{Esr1}-pSI projection promotes male-biased aggression and confirmed that its role in female aggression is not influenced by the sexes of opponents or the estrus cycle.

Sexually disparate synaptic transmission in the VMHv^{Esr1}-pSI projection

Considering the opposing roles of excitatory and inhibitory VMHv-pSI connections in aggression (Figures 3 and 4), we speculated that pSI neurons receive distinct excitatory and/or inhibitory innervation from the VMHv^{Esr1} neurons in males and females. Anatomical differences in the circuitry were first investigated in the two sexes (Figures 6, S4, and S5). The total numbers of GABAergic VMHv^{Esr1} neurons and the total density of VMHv^{Esr1} neurons projecting to the pSI were confirmed as similar in males and females (Figures S4A–S4H). However, by tracing the glutamatergic or GABAergic VMHv neurons with a Cre-dependent, virally encoded Syp: mRuby to target pSI neurons, male mice were confirmed to have a higher density of excitatory presynaptic termini from VMHv neurons in the pSI

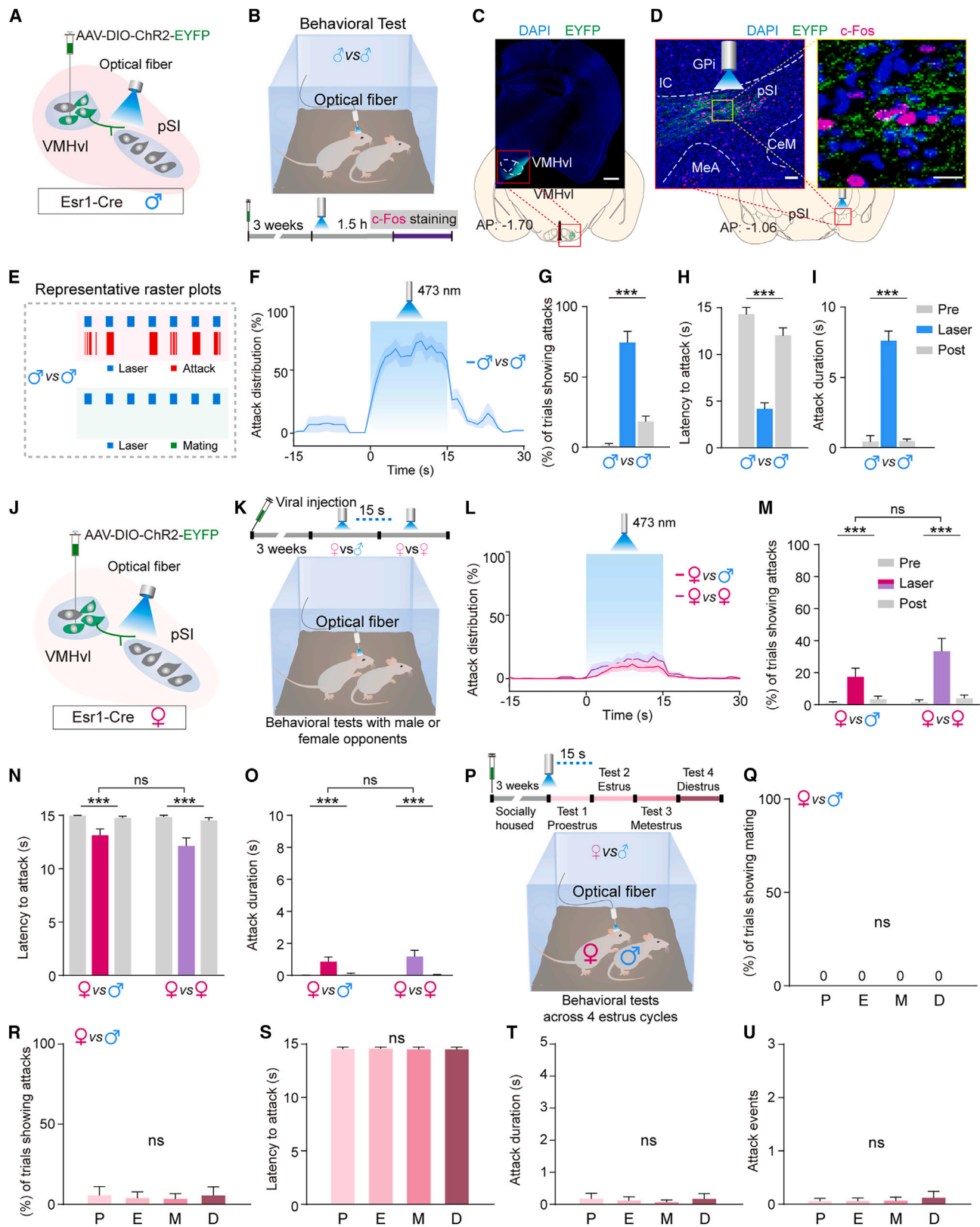
compared with females (Figures 6A–6D, S4I, and S4J), while inhibitory VMHv neurons were noted to send more presynaptic termini to the pSI in females (Figures 6E and S4K). The VMHv-pSI connections of both sexes were further compared using a pseudotyped rabies virus retrograde tracing strategy to trace the connection between aggression-associated VMHv^{Esr1} neurons and pSI^{Thy1} neurons in males and females (Figure 6F). Interestingly, ~80% of the VMHv^{Esr1} neurons projecting to pSI^{Thy1} neurons were glutamatergic in males, while over half of the VMHv^{Esr1} projections were GABAergic neurons in females (Figures 6G–6K).

To validate the functional connections of GABAergic or glutamatergic VMHv-pSI projections, we expressed the Cre-dependent ChR2-EYFP virus in VMHv in *Esr1*-Cre male and female mice (Figure 6L). We next measured the postsynaptic responses of pSI neurons during the optogenetic stimulation of the VMHv^{Esr1} terminals in the pSI (Figures 6L–6V). We then optogenetically recorded evoked inhibitory postsynaptic currents (IPSCs) and EPSCs by independent detection of GABA- or α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/N-methyl-D-aspartate (NMDA)-receptor-mediated currents in acute brain slices, respectively (Figure 6L). We thereby confirmed both excitatory and inhibitory synaptic transmissions in the VMHv^{Esr1}-pSI projections (Figure 6M). The IPSCs and EPSCs of pSI neurons were blocked by tetrodotoxin (TTX) and rescued by 4-aminopyridine (4-AP) (Figures 6N, 6R, and 6S), confirming that VMHv^{Esr1} neurons monosynaptically innervated pSI neurons. Although a similar percentage of pSI neurons was excited or inhibited by VMHv^{Esr1}-pSI projection activation in the two sexes (Figure 6Q), the latency of IPSCs in males was longer than in females (Figures 6O, 6R, and 6S). The peak amplitude of EPSCs in pSI neurons was >2.2-fold larger in males (Figures 6P and 6T), while the peak amplitude of IPSCs in pSI neurons was >2.5-fold larger in females (Figures 6O and 6U). The excitation/inhibition ratio (E/I ratio) was significantly larger in males than in females (Figure 6V). The inhibitory synaptic transmission difference between the two sexes was also

Figure 4. Activation of VMHv^{CaMKII}-pSI projection triggers, while VMHv^{VGAT}-pSI projection inhibits, male aggression

- (A) Schematic of whole-cell electrophysiological recording from pSI neurons when photostimulating the VMHv^{CaMKII}-pSI projections in male mice.
 (B) Representative AAV-CaMKII-ChR2-mCherry infection (red) in VMHv neurons, stained with anti-Esr1 (green) and DAPI (blue). Scale bars, 50 μ m. Right, magnified region within the square. Scale bars, 25 μ m.
 (C) Representative traces of light-evoked EPSCs in pSI neurons by photostimulating the VMHv-pSI projections with or without bath application of the glutamate receptor antagonists 6,7-Dinitroquinoxaline-2,3-dione (DNQX) and 2-amino-5-phosphonopentanoate (AP5).
 (D) Amplitude of evoked EPSCs in control, DNQX+AP5, and washout groups.
 (E) Latency of light-evoked EPSCs in pSI neurons when photostimulating VMHv-pSI projections.
 (F–H) Schematic (F), c-Fos (green) expression (G) of presynaptic termini (red) in the pSI on the stimulated (right) and the unstimulated side (left), and inter-male aggression test (H) after the photostimulation (15 s on/30 s off) in male mice. Scale bars, 100 μ m.
 (I–L) Percentage of trials showing attack (I), latency to attack (J), attack duration (K), and attack events (L) by photostimulation of the VMHv-pSI projection during pre-laser, laser, and post-laser phases in male and female mice.
 (M) Schematic of virus injections and photostimulation of VMHv^{VGAT}-pSI projections in VGAT-Cre male mice.
 (N) Representative image (left) showing the expression of ChR2-EYFP (green) in the VMHv of a VGAT-Cre male mouse. Scale bars, 100 μ m. Right, magnification of the boxed area, and arrowheads indicate the overlap of ChR2 with Esr1. Scale bars, 20 μ m.
 (O) Representative image of ChR2-EYFP (green) terminals in the pSI. Scale bars, 100 μ m.
 (P–W) (P and T) Behavioral paradigms to assess the male (P) and female (T) aggression during the photoactivation of the VMHv^{VGAT}-pSI projections. (Q–U) Distribution of attack episodes interrupted by the photoactivation in males (Q) and females (U). (R and S) Percentage of attack trials interrupted within 3 s (R) and latency to attack interruption (S) by sham/laser stimulation in males. (V and W) Percentage of trials showing attack (V) and attack duration (W) by sham/laser stimulation in females.

See also Figures S2 and S3.



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separately confirmed using whole-cell patch recording from pSI neurons in brain slices of male and female VGAT-Cre mice when optogenetically stimulating the VMHvl^{VGAT} terminals in the pSI (Figure S5A). Larger peak amplitude of IPSCs and a greater proportion of inhibitory responses to pSI neurons indicate that stronger inhibitions were found in females than in males (Figures S5B–S5D).

Taken together, the postsynaptic responses of downstream pSI neurons differ between the sexes. Males have stronger excitatory responses, and females have stronger inhibitory responses.

Sexually dimorphic excitation of pSI neurons by VMHvl^{Esr1} neurons

Given the observed sex differences between excitatory and inhibitory connections of the VMHvl^{Esr1}-pSI projection, we hypothesized that varying activation levels of pSI neurons may result. To validate this, AAV viruses encoding Cre-dependent ChrimsonR were injected into the VMHvl and hSyn-GCamp6m into the pSI (Figure 7A). Fiber photometry was then employed to record the calcium activity of pSI neurons in both sexes during the photoactivation of the VMHvl^{Esr1}-pSI projection (Figures 7B, S6H, and S6I). During photostimulation, pSI neuronal activity showed an increase in both sexes (Figures 7C and 7D). However, neuronal activation in the male pSI was substantially stronger compared with that in the female during photoactivation (Figures 7E–7G). We then used *in vivo* electrophysiological optrodes to record individual pSI neuronal activity during photoactivation (Figures 7H and S6J). Optrode recordings during laser stimulation showed that while males and females showed a similar magnitude of excitation and inhibition (Figures 7I–7P), fewer pSI neurons were activated, and increased numbers were inhibited, in females as compared with males (Figures 7Q–7S). These results suggest activation of the VMHvl^{Esr1}-pSI projection leads to more pSI neuronal excitation in males than in females. To understand how the activity of the VMHvl^{Esr1}-pSI projection or local pSI neurons scales with aggression, the laser intensity-dependent effects on pSI neuronal activity as well as the aggressive behavioral intensity were examined during graded optogenetic activation of the VMHvl^{Esr1}-pSI projection or pan-pSI neurons (Figure S6). The correlation of pSI neuron activity with the activation of the

VMHvl^{Esr1}-pSI projection in males was then examined via injection of AAV viruses encoding ChrimsonR into the VMHvl. Concurrently, hSyn-GCamp6m viruses were injected into the pSI, and the optic fibers were placed into the pSI of male *Esr1*-Cre mice (Figure S6A). Fiber photometry recordings of pan-pSI neurons during escalated photoactivation of the VMHvl^{Esr1}-pSI projection in male mice revealed that pSI neurons were activated in tandem with the enhanced stimulation of the VMHvl^{Esr1}-pSI projection (Figures S6B–S6G). Subsequently, we optogenetically activated the VMHvl^{Esr1}-pSI projection at various laser powers and compared the aggressive behaviors across the increased illumination intensities (Figure S6K). Aggressive behaviors were positively correlated with the intensity of photostimulation in male mice (Figure S6L). To analyze aggressive behaviors during graded optogenetic activation of pan-pSI neurons, we increased laser power in male mice expressed with the AAV-hsyn-ChR2-EYFP in pSI neurons. Aggressive behaviors were again positively correlated with the intensity of photostimulation of pSI neurons in males (Figures S6M–S6O). Together, these results indicate that activation of the VMHvl^{Esr1}-pSI projection triggers more robust neuronal excitation in the pSI of males compared with females, potentially leading to a male bias in aggressive behavior.

DISCUSSION

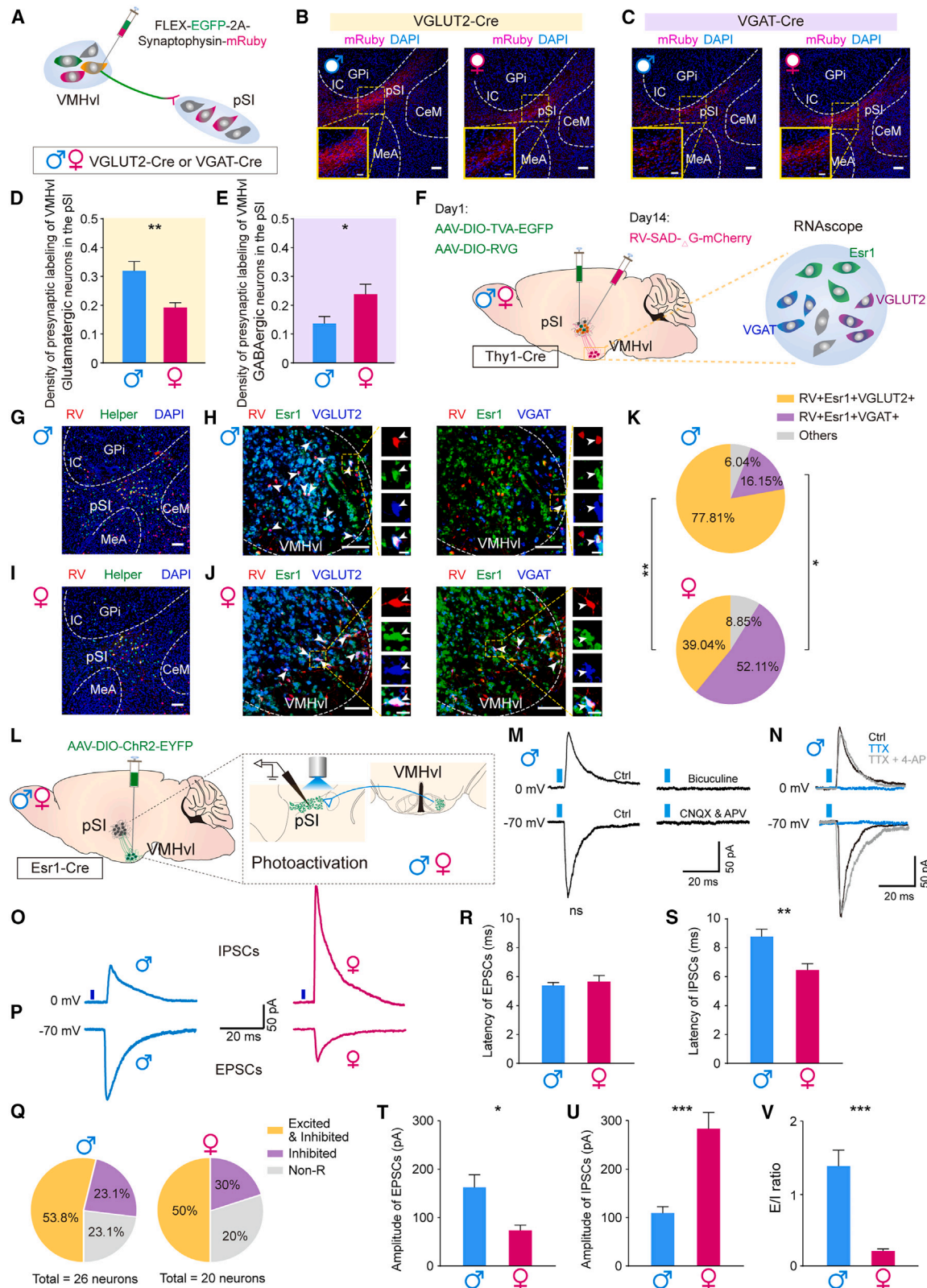
Our study highlights a critical circuit from the VMHvl to the pSI in aggression. The excitation-inhibition balance in the VMHvl-pSI circuit suggests that sex-specific aggression may emerge from interactions between sexually dimorphic (VMHvl) and sexually common (pSI) aggression-eliciting modules.

Parsing the functional targets of the VMHvl in aggression

The VMHvl has emerged as a hub for aggression in mice.^{27,30,38,39} As part of the extended amygdala, the pSI universally controls aggressive behaviors,²⁸ being one of the major output-biased relay regions of the VMHvl.³² Our results showed hypothalamic projections to the amygdalar pSI neurons causally elicited aggression. Although the VMHvl was previously thought to be primarily composed of excitatory neurons, GABAergic cells had also been detected in its lateral border,^{8,36} but with any

Figure 5. Activation of VMHvl^{Esr1}-pSI projection triggers male-biased aggression

(A and B) Strategy for photostimulation of the VMHvl^{Esr1}-pSI projection (A) and behavioral paradigm to test inter-male aggression (B). (C) Representative AAV-DIO-ChR2-EYFP (green) infection in the VMHvl^{Esr1} neurons. Scale bars, 500 μ m. (D) Left, a representative image of AAV-DIO-ChR2-EYFP terminals (green) and c-Fos+ neurons (red) in the pSI of a male mouse after optical activation of the VMHvl^{Esr1}-pSI projection. Right, magnification of the boxed area. Scale bars, 100 μ m, and inset, 50 μ m. (E) Representative raster plots of male attack (upper) and mating behavior (lower) recorded during optogenetic activation of the VMHvl^{Esr1}-pSI projection. (F–I) Attack distribution (F), probability of trials showing attack (G), latency to attack onset (H), and attack duration (I) during photostimulation of the VMHvl^{Esr1}-pSI projection (15 s on/30 s off) in male mice. (J and K) Strategy for photostimulation (J), and behavioral paradigm (K), to assess female-male and female-female aggression induced by photostimulation of the VMHvl^{Esr1}-pSI projection (15 s on/30 s off). (L–O) Distribution of female-male and female-female aggression (L), probability of trials showing attack (M), latency to attack onset (N), and attack duration (O), triggered by photostimulation of the VMHvl^{Esr1}-pSI projection in female mice. (P–U) Strategy and behavioral paradigm for testing the female-male aggression (P), probability of trials showing mating (Q), probability of trials showing attack (R), latency to attack onset (S), attack duration (T), and attack events (U) triggered by photostimulation (15 s on/30 s off) across estrus phases (including proestrus [P], estrus [E], metestrus [M], and diestrus [D]) in females. See also Figure S4.



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potential roles in aggression remaining unknown.^{30,36} We discovered that the GABAergic VMHvl neurons have an opposing role to that of the excitatory VMHvl neurons. Their input to the pSI acts as a “brake” on male aggression, thereby elucidating an initial role for VMHvl inhibitory neurons in social behaviors.

Interestingly, there does appear to be a sex-specific role of inhibitory VMHvl-pSI projections in aggression. While we observed that this projection is effective in suppressing male aggression, its inactivation failed to increase female aggression. Several factors may contribute to the less pronounced effects of the VMHvl^{VGAT}-pSI projection in female aggression including weak excitatory inputs from VMHvl^{Esr1} neurons to pSI neurons in female mice (Figures 6 and 7), a higher threshold of activating pSI for triggering female aggression,²⁸ and the presence of other upstream inhibitory inputs to pSI, such as those of the central amygdala (CeA), zona incerta (ZI), and bed nucleus of the stria terminalis (BNST).²⁸ Thus, the activity of excitatory VMHvl^{Esr1}-pSI inputs may not reach the necessary threshold for triggering aggression in females, even when the VMHvl^{VGAT}-pSI pathway is suppressed. All these factors may serve as redundant dampening mechanisms for female aggression, potentially facilitating evolutionary advantages within social groups.

A hypothalamic-amygdala-midbrain circuit underlying sexually dimorphic aggressive behaviors

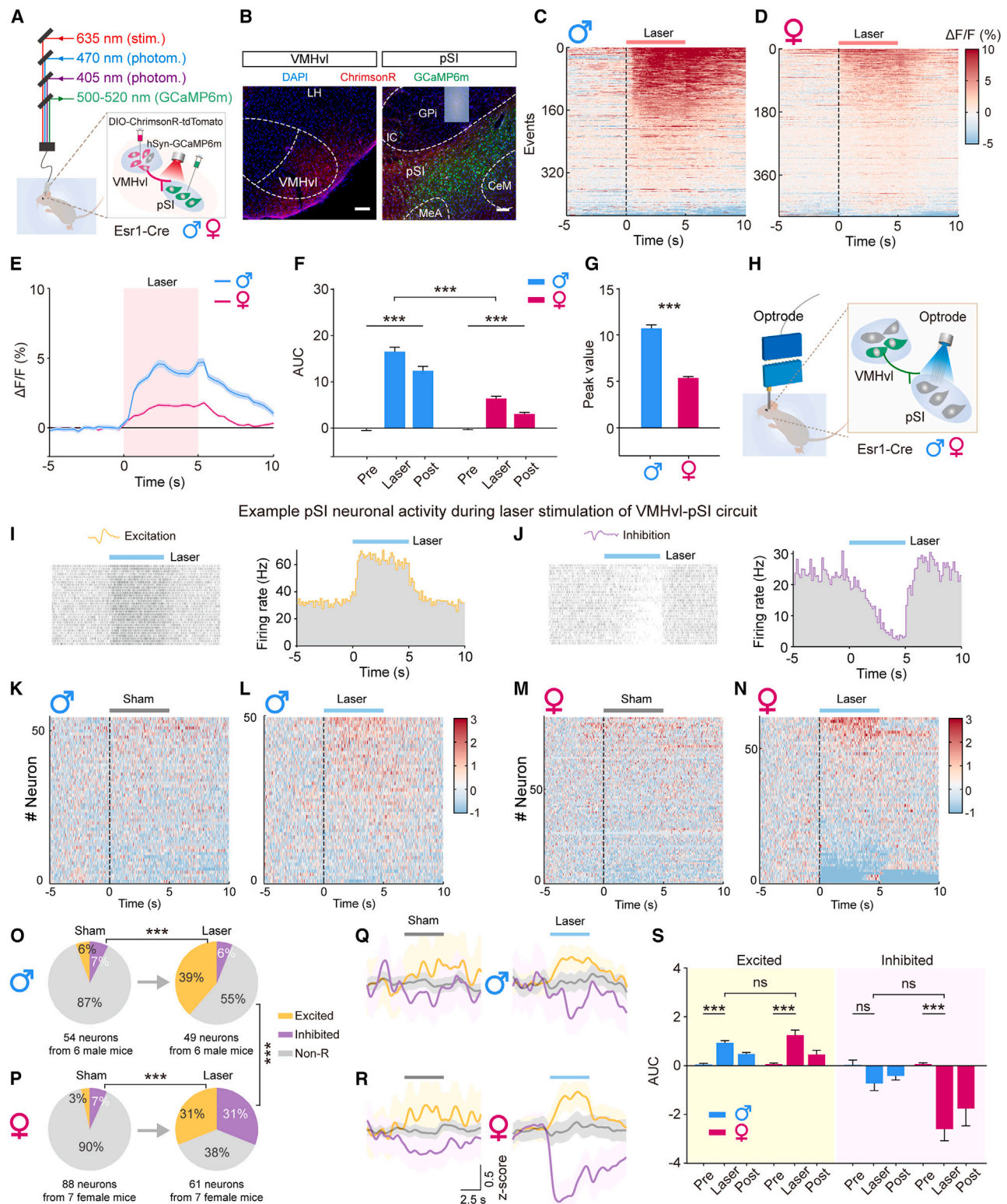
In mammals, circulating sex hormones are essential players in establishing sexually dimorphic aggressive behaviors,^{10,11,40} where such immediate aggressive responses are often reliant on sex-specific neural circuit excitability. Our results reveal that differential excitatory and inhibitory inputs from sexually dimorphic aggression-eliciting modules in the VMHvl to the sexually common aggression-eliciting modules in the pSI regulate male-biased aggressive behavior. The pSI has recently been identified as a

crucial amygdalar region that innervates the PAG to execute aggression.²⁸ In contrast to the sexually dimorphic aggression-eliciting circuits upstream of the pSI, such as those of the VMHvl,^{8,22} activation of the pSI-PAG circuit overrides the requirement for specific environmental conditions for female aggressive behaviors. In humans, functional magnetic resonance imaging shows men have higher extended amygdala activation during strong provocation and anger.⁴¹ In mice, we similarly demonstrated a stronger excitation-biased output from the VMHvl to the pSI in males, indicating that circuit differences that contribute to sexually dimorphic aggression are also present in the extended amygdala of mice.

Many sexually differential factors could be integrated into aggression-relevant circuits, leading to sexually differential aggression in mice. For example, the VMHvl is a crucial hypothalamic region exhibiting substantial sexual dimorphism at molecular, cellular, hormonal, and behavioral levels.^{8,9,30,36} Male VMHvl neurons are transcriptionally and anatomically distinct from those of females.^{8,36,42} Aggression-responsive cells are more widely distributed in the male VMHvl, being more medially concentrated in females. The female VMHvl contains two molecularly distinguishable subregions. Fighting-induced c-Fos were mainly found in the medial zone of VMHvl, whereas mating-induced c-Fos mostly occurred in the lateral zone of VMHvl.^{8,43,44} Male VMHvl cells also exhibit higher activities during aggression than their female counterparts.^{8,30} However, the mechanism underlying the less frequent aggression induction in females upon direct activation of VMHvl cells has remained elusive. Our study sheds light on this issue by demonstrating that VMHvl neurons in males and females provide differential strength in excitatory and inhibitory inputs to the pSI, resulting in male-biased aggression. This finding clarifies sex-biased “hypothalamic aggression” by emphasizing the critical balance between excitatory and inhibitory effects from the VMHvl on

Figure 6. Sexually dimorphic glutamatergic/GABAergic synaptic strength from VMHvl^{Esr1} neurons to the pSI

(A) Anterograde virus tracing for the VMHvl-pSI connections in VGLUT2-Cre or VGAT-Cre male and female mice.
(B and C) Representative synaptophysin-mRuby glutamatergic (B) and GABAergic (C) presynaptic termini (red) expression in the pSI of male (left) and female (right) mice. Scale bars, 100 μ m.
(D and E) Density of mRuby+ glutamatergic (D) or GABAergic (E) presynaptic termini in the pSI of male and female mice (normalized to the mRuby density of VMHvl).
(F) Strategy for identifying the cell type of VMHvl^{Esr1} neurons projecting to the pSI^{Thy1} neurons by retrograde rabies tracing and RNAscope.
(G–J) (G and I) Representative images of rabies virus (red) and helper (green) infection in the pSI of male (G) and female (I) mice. Scale bars, 100 μ m. (H and J) Representative images of the VMHvl showing rabies virus (red) infection and the expression of *Esr1* (green), VGLUT2 (left, blue), or VGAT (right, blue) in male (H) and female (J) mice. Arrowheads indicate co-labeled neurons. Right, magnification of left rectangular region. Scale bars, 20 μ m.
(K) Proportions of VGLUT2 (yellow), VGAT (purple) expressing neurons, and other unidentified neurons (gray) among the VMHvl^{Esr1} neurons projecting to the pSI in male (upper) and female (lower) mice.
(L) Schematic of whole-cell voltage-clamp recordings from pSI neurons in brain slice preparations in response to photostimulation of the VMHvl^{Esr1}-pSI projections in male and female mice.
(M) Representative traces of an IPSC (upper) and an EPSC (lower) evoked in pSI neurons by photostimulation (blue laser) in male mice with (right) or without (left) bath application of the GABAA receptor antagonist bicuculline (top) and the glutamate receptor antagonists CNQX and AP5 (bottom). Scale bars are 20 ms and 50 pA.
(N) Representative traces of IPSCs (upper) and EPSCs (lower) evoked by photostimulation without (control, black) or with bath-application of TTX (blue) or TTX plus 4-AP (gray) in male mice. Scale bars are 20 ms and 50 pA.
(O and P) Representative traces of IPSCs (O) and EPSCs (P) evoked by photostimulation (blue laser) in males (left) and female mice (right). Scale bars are 20 ms and 50 pA.
(Q) Relative proportions of excited (yellow), excited and inhibited (purple), and unaffected (gray) pSI neurons by laser stimulation.
(R–V) Latency of evoked EPSCs (R) and IPSCs (S), the amplitude of evoked EPSCs (T) and IPSCs (U), and E/I ratio (amplitude of EPSCs/IPSCs, V) by photostimulation in male and female mice.
See also Figures S4 and S5.



pSI neuronal excitation across sexes. The VMHvl has over a dozen excitatory cell types, including male-specific cell types.^{36,43} This raises questions about whether similar or different subtypes of excitatory or inhibitory neurons in the VMHvl that connect to pSI neurons occur between the sexes, a topic warranting further investigation.

Neural circuit excitability differentiates sex-specific information in social behaviors

In this study, we found multifaceted sex differences in the VMHvl-pSI projection underlying sexually dimorphic aggressive behaviors. We propose male-biased aggression may be triggered by a simple but efficient circuit logic (Figure S7), where activated VMHvl aggression-responsive neurons transmit stronger excitation and weaker inhibition to the pSI in males than in females (as suggested in Figures 5, 6, and 7). Stronger pSI excitation by the VMHvl in males thereby leads to more potent male aggression (Figures 4, 5, 6, and 7).²⁸

Differences in the excitability of a neural circuit are critical in determining sex-related social behaviors.^{45–48} For example, sex differences in neural excitability have been identified in the medial preoptic area,⁴⁹ anteroventral periventricular nucleus (AVPV),³⁷ and medial amygdala (MeA)⁵⁰ in mice. Dominant male mice show greater synaptic strength in the medial prefrontal cortex and synaptic strengthening of input projections,^{51,52} suggesting synaptic transmission changes underlie male-biased social competition. Notably, excitability differences in brain circuits have been implicated in various brain disorders (e.g., autism spectrum disorders and schizophrenia).^{47,53–55} Autism and schizophrenia patients also display sex-biased pathological aggression,^{56–58} caused by reduced GABAergic signaling.^{3,59,60} Given that the elevated excitatory-inhibitory ratio within the pSI profoundly elicits male-biased aggressive displays, our data may provide crucial insights into how defined changes in the excitatory-inhibitory balance in a specific circuit may affect social interactions in health and disease.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.neuron.2024.06.022>.

ACKNOWLEDGMENTS

We thank Dayu Lin, Nirao M. Shah, Matthew Lee, and Christopher Wood for the discussions and/or reading of the manuscript. We thank Hui-Fang Lou, Shen Lin, Jintao Wu, Shiqi Wang, and Li-Ya Zhu for their technical support. This work was supported by grants from the National Natural Science Foundation (NSFC) of China (82288101, 82090033, and U20A6005 to S.D.; T2293733 and T2293730 to Y.-q.Y.; 32171007 to L.S.; and 32241004 to H.Y.); STI2030-Major Projects (2021ZD0203400 to Y.-q.Y.); Key R&D Program of Zhejiang Province (2024SSYS0017 and 2020C03009 to S.D.; 2022C03034 to Y.-q.Y.); CAMS Innovation Fund for Medical Sciences (2019-I2M-5-057 to S.D.); the Natural Science Foundation of Zhejiang Province (LZ22H090001 to Y.-q.Y.); the Non-profit Central Research Institute Fund of Chinese Academy of Medical Sciences (2023-PT310-01 to Y.-q.Y.).

AUTHOR CONTRIBUTIONS

Z.Z. conducted *in vivo* electrophysiological recordings. K.L., C.S., Y.D., and Q.G. conducted *in vitro* electrophysiological recordings. L.M. conducted *in vivo* fiber photometry recordings. Z.Z., L.M., Q.M., and L.P. performed the immunostaining experiments and behavioral tests. Z.Z., L.M., K.L., and Q.M. analyzed data. Z.Z., L.M., K.L., L.Y., X.X., H.Y., L.-H.Z., Y.L., H.X., X.-m.L., L.S., S.D., and Y.-q.Y. interpreted the results and commented on the manuscript. Z.Z., L.M., S.D., and Y.-q.Y. wrote the manuscript. Z.Z., S.D., and Y.-q.Y. supervised all aspects of the project. The order of co-first authorship was established through a Coin Guessing Match. Both Z.Z. and L.M. contributed equally and have the right to list their name first in their CV.

DECLARATION OF INTERESTS

S.D. is a member of the advisory board of *Neuron*.

(C and D) Heatmaps of calcium signals from pSI neurons during photoactivation (635 nm laser) in male (C) and female (D) mice.
(E–G) Averaged $\Delta F/F$ (E), area under the curve (AUC) (F), and peak $\Delta F/F$ (G) of calcium signals during photostimulation in male and female mice.
(H) Schematic of *in vivo* electrophysiological recordings of multi-unit activity from pSI neurons during photostimulation of the VMHvl^{Esr1}-pSI projections.
(I and J) Examples of neurons excited (I) and inhibited (J) by photostimulation (470 nm) of the VMHvl-pSI projection in male mice.
(K–N) Heatmaps of the pSI neuron activities induced by sham (K and M) and laser stimulation (L and N) in male and female mice.
(O and P) Relative proportions of the excited (yellow), inhibited (purple), and non-responsive (gray) pSI neurons induced by laser stimulation in male (O) and female (P) mice.
(Q and R) Normalized neuronal activity (Z score) of excited, inhibited, and non-responsive pSI neurons before, during, or after photostimulation in male (Q) and female (R) mice.
(S) AUC of firing rate for excited (left), inhibited (right) pSI neurons before (pre), during (laser), or after (post) the photoactivation in male and female mice. See also Figures S6 and S7.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the corresponding author used ChatGPT-4 to proofread the main text. No generative AI tools have been used to produce any new content. The entire article has been reviewed following ChatGPT-assisted spell check. The authors take full responsibility for the content of the publication.

Received: July 29, 2023

Revised: May 13, 2024

Accepted: June 20, 2024

Published: July 16, 2024

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-c-Fos	Calbiochem	Cat# PC38
Rabbit polyclonal anti-Esr1	Millipore	Cat# ab64053
Alexa Fluor 488 conjugate Goat Anti-Rabbit	Invitrogen	Cat# A21206
Cy3-conjugated Goat Anti-Rabbit	Jackson	Cat# 111-165-144
Alexa Fluor 647-conjugated Goat Anti-Rabbit	Invitrogen	Cat# A31573
Bacterial and Virus Strains		
AAV2/2Retro-CMV-Cre-EGFP	Shanghai Taitool Bioscience	Cat# S0231-2R
AAV2/2Retro-CAG-FLEX-Flpo	Shanghai Taitool Bioscience	Cat# S0273-2R
AAV2/2Retro-CMV-EGFP	Shanghai Taitool Bioscience	Cat# S0237-2R
AAV2/9-hEF1a-fDIO-EYFP	Shanghai Taitool Bioscience	Cat# S0253-9
AAV2/9-hEF1a-fDIO-mCherry	Shanghai Taitool Bioscience	Cat# S0553-9
AAV2/9-FLEX-EGFP-2A-Synaptophysin-mRuby	Shanghai Taitool Bioscience	Cat# S0250-9
AAV2/9-hSyn-DIO-jGCaMP7f	Shanghai Taitool Bioscience	Cat# S0588-9
AAV2/9-hSyn-GCaMP6m	Shanghai Taitool Bioscience	Cat# S0471-9
AAV2/9-hEF1a-DIO-hChr2(H134R)-EYFP	Shanghai Taitool Bioscience	Cat# S0199-9
AAV2/9-hEF1a-fDIO-hChr2(H134R)-EYFP	Shanghai Taitool Bioscience	Cat# S0129-9
AAV2/9-hSyn-FLEX-ChrimsonR-mCherry	Shanghai Taitool Bioscience	Cat# S0739-9
AAV2/9-hSyn-GtACR1-EGFP	Shanghai Taitool Bioscience	Cat# S0450-9
AAV2/9-hEF1a-DIO-hM3d(Gq)-mCherry	Shanghai Taitool Bioscience	Cat# S0192-9
AAV2/9-hEF1a-DIO-hM4d(Gi)-mCherry	Shanghai Taitool Bioscience	Cat# S0193-9
AAV2/9-hEF1a-fDIO-hM4d(Gi)-mCherry	Shanghai Taitool Bioscience	Cat# S0336-9
AAV2/9-mCaMKII-hChr2 (H134R)-mCherry	Shanghai Taitool Bioscience	Cat# S0166-9
AAV2/9-mCaMKII-hM4Di (Gi)-EGFP	Shanghai Taitool Bioscience	Cat# S0500-9
Rabies viruses SAD-OG-mCherry (EnvA)	BrainVTA, Wuhan	N/A
AAV-DIO-G	BrainVTA, Wuhan	Cat# AAV-902
AAV-DIO-TVA-EGFP	BrainVTA, Wuhan	Cat# AAV-903
Rabies Δ G-GFP-EnvA viruses	BrainVTA, Wuhan	Cat# RV-306
Chemicals, Peptides, and Recombinant Proteins		
Clozapine N-oxide (CNO)	Sigma-Aldrich	Cat# C0832
DAPI	Sigma-Aldrich	Cat# D9542
Critical Commercial Assays		
RNAscope® Multiplex Fluorescent Reagent Kit v2	ACD Biosciences	Cat# 323100
Experimental Models: Organisms/Strains		
C57BL/6	Slackom company, Shanghai	N/A
Esr1-Cre mice	The Jackson Laboratory	Stock No: 017913
Thy1-Cre mice	The Jackson Laboratory	Stock No: 006143
Oligonucleotides		
RNAscope Mm- Slc32a1-C1 probe	ACD Biosciences	Cat# 319191
RNAscope Mm-Esr1-C2 probe	ACD Biosciences	Cat# 496221-C2
RNAscope Mm-Slc17a6-C3 probe	ACD Biosciences	Cat# 319171-C3
Hardware, Software and Algorithms		
MATLAB	MathWorks	https://www.mathworks.com/products.html ; RRID:SCR_001622

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
GraphPad Prism 6/8	GraphPad Software	https://www.graphpad.com/scientificsoftware/prism/ ; RRID: SCR_002798
VS120 Virtual Slide Microscope	Olympus Scientific Solutions	https://www.olympus-lifescience.com/en/solutions-based-systems/research-slide-scanner/
ImageJ	NIH	https://imagej.nih.gov/ij/index.html ; RRID:SCR_003070
OmniPlex Neural Recording Data Acquisition Systems	Plexon	https://plexon.com/products/omniplex-software/ ; RRID: SCR_014803
Offline Sorter	Plexon	www.plexon.com/products/offline-sorter ; RRID:SCR_000012
NeuroExplorer	Plexon	https://plexon.com/products/neuroexplorer/ ; RRID:SCR_001818
465/470 nm LED	Inper, Hangzhou, China	https://www.inper.com/
635 nm LED	Inper, Hangzhou, China	https://www.inper.com/
Implantable Optical Fibers	Inper, Hangzhou, China	https://www.inper.com/
16 tungsten microwires, 35- μ m diameter	California Wire, US	N/A
Behavioral Analysis Code, MATLAB	This paper	N/A
Fiber photometry system	Thinkertech, Nanjing, China	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shumin Duan (duanshumin@zju.edu.cn).

Materials availability

All expression vectors reported in this study are available upon request.

Data and code availability

All raw datasets are available upon request. This study did not generate new code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Male Thy1-Cre, VGAT-Cre, C57BL/6J (C57), Esr1-2a-Cre (Esr1-Cre, #017913), and BALB/c mice of both sexes were used. For functional manipulation and *in vivo* recording experiments, 8–20-week-old mice were used. For *in vitro* slice recording experiments, the virus was injected into brain regions when the mice were 6–7 weeks old. Mice were housed under $22 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity with food and water *ad libitum*. Animal experiments were conducted following the approval of the ethics committee and the Guidelines for the Care and Use of Laboratory Animals of Zhejiang University.

METHOD DETAILS

Viral Injections and Stereotaxic Surgery

AAV2/2Retro Plus-hSyn-Cre-EGFP (1.22×10^{13} genomic copies per ml), AAV2/2Retro-CAG-FLEX-Flpo (1.66×10^{13} genomic copies per ml), AAV2/2Retro-CMV-EGFP (1.32×10^{13} genomic copies per ml), AAV2/9-hEF1a-fDIO-EYFP (1.53×10^{13} genomic copies per ml), AAV2/9-hEF1a-fDIO- mCherry (1.25×10^{12} genomic copies per ml), AAV-FLEX-EGFP-2A-Synaptophysin-mRuby (1.55×10^{13} genomic copies per ml), AAV2/9-hSyn-DIO-jGCaMP7f (2.0×10^{13} genomic copies per ml), AAV2/9-hsyn-GCaMP6m (2.53×10^{13} genomic copies per ml), AAV2/9-hEF1a-DIO-hChR2(H134R)-EYFP (2.95×10^{13} genomic copies per ml), AAV2/9-hEF1a-fDIO-hChR2(H134R)-EYFP (1.93×10^{13} genomic copies per ml), AAV2/9-hSyn-FLEX-ChrimsonR-mCherry (1.8×10^{13} genomic copies per ml), AAV2/9-hSyn-hGtACR1-EGFP (1.35×10^{13} genomic copies per ml), AAV2/9-hEF1a-DIO-hM3d(Gq)-mCherry (1.66×10^{13} genomic copies per ml).

genomic copies per ml), AAV2/9-hEF1a-DIO-hM4d(Gi)-mCherry (1.82×10^{13} genomic copies per ml), AAV2/9-hEF1a-fDIO-hM4d(Gi)-mCherry (1.18×10^{13} genomic copies per ml), AAV2/9-mCaMKII-hChR2 (H134R)-mCherry (2.03×10^{13} genomic copies per ml), AAV2/9-mCaMKII-hM4Di(Gi)-EGFP (1.86×10^{13} genomic copies per ml), were provided by Taitools Co. (Shanghai, China). AAV-CAG-DIO-TVA-EGFP (AAV2/9, 1.7×10^{13} genomic copies per ml), AAV-CAG-DIO-RG (AAV2/9, 6.8×10^{12} genomic copies per ml), and EnvA-pseudotyped, glycoprotein (RG)-deleted and DsRed-expressing rabies virus (RV-EvnA-DsRed, RV) (5.0×10^8 genomic copies per ml) were provided by BrainVTA Co. (Wuhan, China).

Mice were anesthetized with sodium pentobarbital (1%) before stereotaxic injection. A volume of 50–300 nL virus solution (depending on the titer and measured expression strength) was prepared, and 0.05–0.1 μ L was injected into each location at 0.01 μ L/min, not removing the needle until 5–10 min after the end of infusion to allow for diffusion of the virus. A heating pad ensured the maintenance of core body temperature at 36°C. The coordinates of viral injection sites were: VMHvl (AP, -1.70 mm; ML, -0.7 mm; DV, -5.8 mm), pSI (AP, -0.90 mm; ML, -2.2 mm; DV, -4.5 mm) and PAG (AP, -4.70 mm; ML, -0.7 mm; DV, -2.8 mm). The coordinates of the cannula or optical fiber placement sites in the PAG, VMHvl, or pSI were placed at 200–400 μ m above the virus injection site. Histology was obtained and verified from all test animals. For the analyzed physiological and histology results, the initial identification of injections or fiber/electrode placements that hit the target was done blind to the physiological results. The supplemental table in the manuscript includes animals with both successful (“Included”) and unsuccessful (“Excluded”) experiments.

Anterograde and Retrograde Viral Tracing

To label projection termini of VMHvl^{Esr1} neurons in the pSI, a Cre-dependent, and virally encoded mCherry fused to the synaptic vesicle protein synaptophysin (Syp: mRuby) was unilaterally targeted to VMHvl^{Esr1} neurons in male or female *Esr1-2a-Cre* mice (diluted 1:1, 50 nL). After waiting 3–4 weeks to allow Syp: mRuby to be anterogradely transported to the pSI, the sections of the pSI and VMHvl were collected. The density of mRuby+ termini was scored using ImageJ software and the density of the presynaptic termini was normalized by the number of mRuby+ signals in VMHvl neurons.

To explore the sexually dimorphic projection termini with different excitability, AAV-FLEX-EGFP-2A-Synaptophysin-mRuby (diluted 1:1, 50 nL/site) was injected into VGLUT2-Cre or VGAT-Cre male and female mice. After 3 weeks, the sections of the VMHvl and the downstream regions of the VMHvl were collected to evaluate the Syp: mRuby+ presynaptic termini in these regions. The density of mRuby+ termini was scored using ImageJ software and the density of the presynaptic termini was normalized by the number of mRuby+ signals in VMHvl neurons.

Retrograde tracing of the connection between VMHvl neurons and pSI^{Thy1} neurons of the two sexes was conducted. Specifically, a pseudotyped rabies virus retrograde tracing strategy was used to trace the monosynaptic presynaptic neurons to the pSI^{Thy1} neurons in males and females. On day 1, the Cre-dependent AAV virus carrying TVA, RG, SADΔG-EGFP (EnvA) was injected into the pSI (100 nL/site) of *Thy1-Cre* mice, and on day 15, the pseudotyped rabies virus was injected at the same site (50 nL/site). The extent of viral infection was first confirmed in the pSI. The VMHvl neurons that were retrogradely labeled in both male and female mice were then observed. To investigate whether the RV+ labeled VMHvl neurons were differently co-localized with *Esr1*, VGLUT2, and VGAT in two sexes, RNAscope for *Esr1*, VGLUT2, and VGAT for the VMHvl were investigated.

The monosynaptic presynaptic neurons to pSI^{PAG} neurons were retrogradely traced using a pseudotyped rabies virus retrograde tracing strategy for validating the connection between VMHvl neurons and pSI^{PAG} neurons in male mice. On day 1, AAV2/2-Retro-CMV-Cre-EGFP⁶¹ (100 nL/site) was first injected into the PAG to retrogradely express Cre in pSI neurons. Then the Cre-dependent AAV carrying TVA, RG, SADΔG-EGFP (EnvA) virus, and rabies virus (50 nL/site) were injected into the pSI at the same site to trace the upstream neurons. On day 15, the pseudotyped rabies virus was injected at the same pSI site. The extent of viral infection was checked in the pSI and mCherry+ VMHvl neurons were examined in male and female mice.

To reveal the outputs from the pSI to VMHvl, a retrograde retro-AAV expressing Cre recombinase was injected into the PAG (AAVRetro-Cre) (60 nL/site), and an anterograde AAV expressing ChR2 virus (AAV-DIO-ChR2) was injected into the pSI (100 nL/site). The postsynaptic terminals of pSI^{PAG} neurons were then examined in the VMHvl.

Behavioral Measurements and Video Analysis

Males and females with or without aggressive behaviors were used for all analyses. The behavioral parameters for aggression tests included the probability of trials with an induced attack, the number of attacks, latency to the first attack, and the total duration of the attack. Aggressive behaviors were scored and counted incorporating biting behavior, tussling, aggressive allogrooming, and pushing in both males and females. We realized that male and female mice bite the conspecifics on different parts of the body, nevertheless, all biting behaviors, regardless of the attack target or opponent and including all the aggression that happened in males and females, were counted. Aggression in our experiment included various social-contextual conditions. Adult virgin males or females were used as test mice. These were singly housed for two weeks or socially housed before the behavioral test. During the test, the mice were introduced to a novel cage or acted as a resident in their home cage for the aggression test. The male mating test ran for 15 min and was videotaped and scored for mounting and mounting with a pelvic thrust.

All videos were recorded at 30 fps, and manual annotation was performed by an observer blind to experimental conditions. The annotations were then processed and analyzed using customized MATLAB programs - Scorevideo to characterize and quantify behavioral episodes as previously described.⁷

HM4Di-mediated Neural Silencing and HM3Dq-Mediated Neural Activation

To activate VMHv^{Esr1} neurons, the AAV-hSyn-DIO-hM3Dq-mCherry (a Cre-dependent pharmacogenetic activating virus, diluted 1:3, 70 nL/side) was directly used to unilaterally infect the VMHv neurons in Esr1-Cre mice of both sexes. Three weeks after the injection, mice were *I.P.* injected with saline on day 1 and then CNO (0.5 mg/kg) on day 2. Thirty minutes after each injection, an adult mouse (20–30 g) was introduced for ~15 minutes to evaluate the aggression level of the male. After the behavioral test, the virus expression in the VMHv and the c-Fos expression in the VMHv and downstream regions of VMHv were examined beyond CNO (0.5 mg/kg) *I.P.* injection into the mice while they were solitary in their home cage.

To examine the necessary function of the VMHv^{CaMKII} neurons in aggression, the CaMKII-hM4Di-mCherry virus was bilaterally injected into the VMHv (100 nL/side) to inhibit excitatory VMHv^{CaMKII} neurons by *I.P.* injection of CNO (1 mg/kg) and the aggressive behavior before and after injection was examined.

To test the necessary function of the VMHv^{VGAT} neurons in aggression, the Cre-dependent DIO-hM4Di virus (80 nL/side) was bilaterally injected into the VMHv of VGAT-Cre female mice. Aggression was recorded for 15 min after chemogenetically inhibiting the VMHv^{VGAT} neurons that project to the pSI in the females by *I.P.* injection of CNO (2 mg/kg) or saline. The aggressive behavior before and after injection was then examined.

To test the necessary function of the inhibitory VMHv^{VGAT}-pSI in aggression, the AAVRetro-DIO-Flpo virus (diluted 1:1, 100 nL/side) was bilaterally injected into the pSI and the AAV-fDIO-hM4Di virus (80 nL/side) was bilaterally injected into the VMHv of VGAT-Cre male and female mice. Aggression was recorded for 15 min after chemogenetically inhibiting the VMHv^{VGAT} neurons that project to the pSI in the male by *I.P.* injection of CNO (2 mg/kg) or saline. The aggressive behavior before and after injection was examined.

GtACR1 Mediated Neural Inhibition

To quantify the contributions of pSI neurons for VMHv-mediated aggression, pSI neurons were inhibited during the aggression mediated by VMHv^{Esr1} neurons to test whether VMHv-induced aggression was blocked. AAV2/9-hEF1a-DIO-hM3d(Gq)-mCherry virus was first unilaterally injected into the VMHv (60 nL/side) of Esr1-Cre male mice to trigger aggression. Then, the pSI neurons were bilaterally inhibited by AAV2/9-hSyn-hGtACR1-EGFP (100 nL/side) with the photostimulation (40 Hz blue laser, 0 mW or 5.0 mW, 15 s on/30 s off, ~10 min for each mouse) during the aggression induced by chemogenetic activation of VMHv^{Esr1} neurons after *I.P.* injection of CNO (0.5 mg/kg).

Chr2 Mediated Neural Activation

For *in vivo* optogenetic manipulation in awake-behaving animals, an optical fiber cannula (200 μ m) was implanted into the pSI. After injection, mice were allowed 3–4 weeks of recovery. To acutely manipulate the VMHv-pSI projection, an optical fiber cannula (200 μ m) was implanted into the pSI. The fiber was connected to a laser source using an optic fiber sleeve. The power of the blue laser (473 nm) was measured at the tip of the optic fiber. Randomly-selected socially or singly housed adult male, or female mice were introduced into the subject's home cage, one at a time and each for 10–20 min. The light intensity was the same as that optimized for eliciting an attack on previous testing days, and the neural activation was assessed by c-Fos staining. Mice with missed injections or cannula locations were excluded. Control mice expressing only EYFP/mCherry underwent the same procedure and received the same intensity of laser stimulation.

To acutely manipulate the VMHv-pSI projection, C57 mice of both sexes were unilaterally stereotaxic injected with AAV-CaMKII-hChr2 (H134R)-mCherry virus (diluted 1:3, 70 nL) into the VMHv, with control animals identically injected with AAV-CaMKII-mCherry (diluted 1:3, 60 nL). To investigate the role of the VMHv^{CaMKII}-pSI projection in inter-male aggression, an adult male intruder was introduced to the cage of a male subject mouse, and aggression and mating behaviors were assessed during photoactivation of the VMHv-pSI projection (40 Hz, 3.0 mW, 15 s on/30 s off, ~10 min for each mouse).

To further clarify the necessity of the pSI projection for expressing VMHv-pSI-controlled aggressive behavior, the pSI was silenced and whether the aggression induced by activating the VMHv-pSI projection changed was investigated. First, a mixture of AAVRetro-DIO-Flpo virus and DIO-hM4D virus (mixing ratio = 2:1, 100 nL/side) was injected into the pSI of Thy1-Cre mice, followed by injection of fDIO-Chr2 virus (80 nL/side) into the VMHv. Beyond chemogenetically inhibiting pSI^{Thy1} neurons in the male by *I.P.* injection of CNO (1 mg/kg) or with saline as control, aggression was recorded for 15 min and analyzed. Next, the projection from VMHv to the pSI was activated by optogenetically stimulating the VMHv^{Thy1}-pSI projection (40 Hz, 3.0 mW, 15 s on/30 s off, ~15 min for each mouse) and the inter-male aggressive behavior before and after *I.P.* injection of saline or CNO were examined.

To address whether the VMHv was involved in pSI-induced aggression, pSI^{PAG} neurons were optogenetically activated and VMHv neurons were inhibited. Any effects upon pSI-induced aggression were then observed. Specifically, CaMKII-hM4Di-mCherry virus was first bilaterally injected into the VMHv (70 nL/side) and excitatory VMHv^{CaMKII} neurons were inhibited by *I.P.* injection of CNO (1 mg/kg), and the aggressive behavior before and after injection of CNO were examined. Then, the aggression induced by optogenetic activation of pSI^{PAG} neurons (40 Hz, 3.0 mW, 15 s on/30 s off, ~15 min for each mouse) before and after the pharmacogenetic inhibition of the VMHv neurons (CNO: 1 mg/kg) was examined.

To acutely activate the VMHv^{Esr1}-pSI projection in males and females, Esr1-Cre mice of both sexes were unilaterally stereotaxically injected with AAV-DIO-Chr2-EYFP virus (diluted 1:4, 50 nL) into the VMHv. To check whether the threshold-dependent effects

for aggression intensity occurred during optogenetic stimulation, the VMHvl-pSI projection was optogenetically stimulated across graded illumination intensities (from 0 mW to 3.0 mW) during inter-male aggression and other aggressive behaviors, including comparisons of varied laser powers.

To analyze aggressive behaviors when activating the VMHvl^{Esr1}-pSI projection in females at different estrous cycle stages, the daily monitoring of estrous cycle stages in female mice was initiated two weeks after injecting the virus. This process involved gently flushing the vaginal canal with 15 mL of PBS, then transferring the flush to a microscope slide and promptly examining it under a brightfield microscope. During different stages of the estrous cycle, namely proestrus, estrus, metestrus, or diestrus cycles, the VMHvl^{Esr1}-pSI projection was optogenetically activated in mice to investigate its influence on aggression in females.

To analyze aggressive behaviors during graded optogenetic activation of pan-pSI neurons *in vivo*, the AAV-hsyn-ChR2-EYFP virus (60 nL, unilaterally) was injected into the pSI in male C57 mice. Then, pSI neurons were photoactivated with increasing laser power (from 0 mW to 3.2 mW), with aggressive behaviors then compared across the varying laser powers.

To acutely activate the VMHvl^{VGAT}-pSI projection in males, VGAT-Cre male mice were bilaterally stereotaxic injected with the AAVRetro-DIO-Flpo virus (100 nL/side) into the pSI, followed by injection of the fDIO-ChR2 virus (100 nL/side) into the VMHvl. The projection from VMHvl^{VGAT} neurons to the pSI was bilaterally activated through photostimulation (40 Hz, 5.0 mW, 15 s on/30 s off, ~15 min for each mouse). Aggression was recorded for ~15 minutes and analyzed.

Slice Electrophysiology

Mice (8–10 weeks) were deeply anesthetized with pentobarbital sodium (100 mg/kg i.p.) and decapitated. The whole brain was quickly dissected into ice-cold oxygenated (95% O₂ and 5% CO₂) cutting solution containing (in mM): 228 sucrose, 10 D-glucose, 26 NaHCO₃, 1 NaH₂PO₄, 2.5 KCl, 3 MgCl₂, 0.5 CaCl₂ (pH 7.3–7.4; ~300 mOsm). The brain was then cut coronally into 300-μm slices on a microtome (VTA-1200S; Leica). Slices containing the pSI were transferred to an incubation chamber filled with artificial cerebrospinal fluid containing (in mM): 125 NaCl, 2.5 KCl, 2 MgCl₂, 2 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 D-glucose (pH 7.3–7.4; ~300 mOsm) bubbled with 95% O₂ and 5% CO₂ and incubated for at least 1 h at room temperature (24–26°C). The slices were transferred to a recording chamber on the stage of a fluorescence microscope (BX51WI; Olympus). Patch electrodes were pulled from borosilicate capillaries (BF150-86-10; Sutter Instruments) having impedances of 3–5 MΩ when filled with intracellular solution containing (in mM) 135 K-gluconate, 6 NaCl, 10 HEPES, 0.5 EGTA, 10 Na₂-phosphocreatine, 4 Mg-ATP, 0.3 Na₂-GTP (pH:7.2, adjusted with KOH; 290–300 mOsm) for recording pSI-PAG neuronal action potentials. For EPSC recordings, pipettes were filled with the solution of the following components (in mM): 135 CsMeSO₃, 10 HEPES, 0.5 EGTA, 3.3 QX-314, 4 Mg-ATP, 0.3 Na₂-GTP, 8 Na₂-phosphocreatine (pH 7.2 adjusted with CsOH; 290–300 mOsm). Recordings were made using a MultiClamp 700B amplifier (Molecular Devices) and Digi-data 1320A interface (Molecular Devices). Neurons that expressed EYFP/mCherry/retrobeads fluorescence were identified and recorded. Signals were low-pass filtered at 10 kHz and digitized at 10 kHz (Micro3 1401, Cambridge Electronic Design). Data were acquired and analyzed using Spike2 7.04 Clampfit 10 software (Cambridge Electronic Design).

To characterize the connections from the VMHvl neurons to the pSI^{PAG} neurons in males, a retrograde Retrobeads or AAVRetro-EYFP virus was first injected into the PAG, and whole-cell patch-clamp was applied to record Retrobeads/EYFP-expressing neurons in the pSI of wild-type males (n = 5 mice). To identify the functional connection of the VMHvl-pSI^{PAG} pathway, action potentials of pSI^{PAG} neurons were recorded when the axon projections from the VMHvl to the pSI^{PAG} neurons were stimulated. The light-evoked EPSCs of Retrobeads/EGFP+ and Retrobeads/EGFP negative neurons were recorded.

To record the light-evoked IPSCs/EPSCs in mice, neurons in the pSI were patched and tested for the effects of photostimulation. To evoke EPSCs or IPSCs, brief (1 ms) 473-nm blue laser stimulation was applied using an X-Cite 110 LED (Excelitas Technologies). The blue laser was focused on the back aperture of the objective to achieve full-field illumination. The IPSCs and EPSCs were recorded at holding potentials of 0 mV and −70 mV in acute brain slices. DNQX (20 μM) and APV (50 μM) (Sigma-Aldrich) were bath-applied to block AMPA, kainite, and NMDA receptors during voltage-clamp recordings of photostimulation-induced excitatory current responses. Bicuculline (40 μM) was bath-applied to block GABAA receptors during voltage-clamp recordings of photostimulation-induced inhibitory current responses.

To check the excitatory response of the CaMKII neurons in the VMHvl, the AAV-CaMKII-ChR2-mCherry virus was expressed in the VMHvl of C57 male mice, and IPSCs and EPSCs in pSI neurons were optogenetically evoked with laser stimulation (3.0 mW), allowing independent detection of both GABA- and glutamate-receptor-mediated currents. The peak amplitude and latency of EPSCs of pSI neurons elicited by optogenetic activation of the VMHvl^{CaMKII}-pSI projection were analyzed.

To measure the postsynaptic responses from VGAT+ VMHvl neurons to the pSI, virally-encoded Cre-dependent ChR2-EYFP were targeted to VMHvl neurons of VGAT-Cre males and females, and IPSCs and EPSCs in pSI neurons were optogenetically evoked with blue laser stimulation (8.4 mW), allowing independent detection of both GABA- and glutamate-receptor-mediated currents. The latency and peak amplitude of IPSCs of pSI neurons elicited by optogenetic activation of the VMHvl^{VGAT}-pSI projection were compared in male and female mice.

To measure the postsynaptic responses from Esr1+ VMHvl neurons to the pSI, virally-encoded Cre-dependent ChR2-EYFP was targeted to VMHvl neurons of Esr1-Cre males and females, and IPSCs and EPSCs in pSI neurons were optogenetically evoked with different intensities of laser stimulation (0 mW, 2.0 mW, and 8.4 mW). The IPSCs and EPSCs were recorded, allowing independent detection of both GABA- and glutamate-receptor-mediated currents. TTX (0.5 μM) and 4-AP (100 μM) were bath-applied during voltage-clamp recordings of photostimulation-induced inhibitory and excitatory current responses to block APs and inhibit network

activity. The probabilities of pSI neurons responding to VMHvl activation in males and females, latency, and peak amplitude of EPSCs and IPSCs of pSI neurons elicited by optogenetic activation of the VMHvl^{Esr1}-pSI projection were compared.

Analysis of Electrophysiological Parameters

All electrophysiological data were analyzed with ClampFit 10 (Molecular Devices) and Mini analysis 6.0 (Synapsoft). The projected positions of these neurons in a reference brain atlas showed that they were tightly clustered within the pSI, with no systematic difference between the two sexes.

Fiber Photometry

The fiber photometry system (Thinker Tech Nanjing Biotech Ltd, Nanjing, China) was used to record calcium signals. Before fiber photometry recording, the GcaMP virus was expressed in the targeted region of the mice. The ferrule was affixed with a skull penetrating M1 screw and dental acrylic. To enable AAV expression and recovery, mice were housed individually for at least 10 days after virus injection. To record fluorescence signals, the beam from a 488-nm laser (OBIS 488LS; Coherent, Santa Clara, CA, USA) was reflected with a dichroic mirror and focused with a 10× objective lens (NA = 0.3; Olympus, Tokyo, Japan). An optical fiber (230 μm OD, NA = 0.37; 2 m long) guided the light between the commutator and the implanted optical fiber. To minimize GCaMP bleaching, the laser power at the tip of the optical fiber was adjusted to a low level (0.03–0.04 mW). The GcaMP fluorescence was filtered with an EYFP bandpass filter and collected by a photomultiplier tube (R3896; Hamamatsu Photonics). An amplifier converted the photomultiplier current output to a voltage signal, which was further filtered through a low-pass filter (40 Hz cut-off; Brownlee 440). The analog voltage signals were digitized at 500 Hz (Power 1401 digitizer, Cambridge Electronic Design) and sampled with software (TDMS). Fiber-photometric recording data were exported as MATLAB files for further analysis, and fiber photometry-related aggression behavioral data were analyzed using MATLAB. All the raw fluorescence data (F) were smoothed with a moving average filter (10-ms span) and then segmented and aligned to the onset of behavioral events within individual trials or bouts. The fluorescence change values ($\Delta F/F$) were calculated as $(F - F_0)/(F_0 - V_{\text{offset}})$, where F_0 is the baseline fluorescence signal averaged over a 2-s/4-s time-window prior to a trigger event, and V_{offset} is the fluorescence signal recorded before the cannula was connected to the optical fiber above the VMHvl. $\Delta F/F$ values are presented as heatmaps or average plots with the shaded area indicating the SEM.

To investigate the neuronal activity of the VMHvl^{VGAT} neurons in the different social behaviors, the AAV-hSyn-DIO-jGCaMP7f (60 nL/side) was injected in the VMHvl of VGAT-Cre males with an optical fiber (230 μm OD, 0.37 NA) placed in a ceramic ferrule (2.5 mm OD, 126 μm ID) and inserted toward the VMHvl. The calcium signals in the VMHvl^{VGAT} neurons were recorded when the VGAT-Cre male mice encountered a male intruder for ~30 min. The average $\Delta F/F$ during aggressive behaviors, sniff without an attack (a sniff not followed by an attack within 4 s), and sniff preceding attack were calculated based on the peri-stimulus time histograms (PSTHs). The non-parametric Wilcoxon signed-rank test or t-test was used to determine whether the AUC per second or peak values of $\Delta F/F$ before, during, or after events were significantly different from control events.

To test how the pan-pSI neurons respond to the VMHvl^{Esr1}-pSI projection, the AAV-DIO-ChrimsonR-tdTomato virus (60 nL/side) was injected in the VMHvl of Esr1-Cre male and female mice and the optical fiber then implanted in the pSI. The neuronal activity of pSI neurons was recorded upon the photoactivation of the VMHvl^{Esr1}-pSI projection with the red laser (635 nm, 0 mW, 1.1 mW, or 3.5 mW) without an intruder. The average $\Delta F/F$ during photostimulation of the projections from VMHvl^{Esr1} neurons to the pSI based on the PSTHs were calculated. The non-parametric Wilcoxon signed-rank test or t-test was used to determine whether the AUC or peak values of $\Delta F/F$ before (pre), during (laser), or after (post) the laser stimulation were significantly different.

Single-unit Recording and Data Analysis

To clarify whether the distinct extent of VMHvl-pSI projection activity determines aggression of two sexes *in vivo*, *in vivo* electrophysiological optrodes were used to record from pSI neurons when optogenetically activating the VMHvl^{Esr1}-pSI projection. Modified electrode arrays were implanted dorsal to the pSI. These consisted of 32 single microwires (California Fine Wire), and an optical fiber (200 μm) glued to the middle of the bundle of electrodes. One ground wire was then soldered to a 32-channel connector (Omnetics Connector). Mice were allowed to recover for at least 7 days, and then the electrodes were connected to a 32-channel preamplifier head-stage (Plexon). During the recording sessions, all signals recorded from each electrode were amplified, and filtered between 0.1 Hz and 10 kHz, with spike waveforms digitized at 40 kHz. Spikes were sorted using Offline Sorter software (Plexon). Units were accepted only if a distinct cluster was visible in a two-dimensional plot of the two largest principal components. Neurons with a mean firing rate >0.5 Hz were included in the analysis.

To understand whether VMHvl-pSI activity can be employed as an aggressive threshold variable determining sex-specific aggression, *in vivo* electrophysiological optrodes were used to directly record from pSI neurons in male and female mice when optogenetically activating the VMHvl^{Esr1}-pSI projection with blue lasers (473-nm) of 0, and 4.8 mW in the sham and stimulation group (laser intensities measured at the laser source rather than the tip of the optrode). In total, 6 male and 7 female Esr1-Cre mice were implanted with electrodes and used for data collection. pSI neuronal activity was analyzed using MATLAB. If a given neuron had a significant response to the events (e.g., laser stimulation), it displayed a more reliable response across trials. The average response across trials was calculated based on Z-scored PSTHs. For each recorded unit, PSTHs were Z-score transformed by subtracting the mean firing rate and dividing the standard error of each unit's firing rate. To test the significance of firing rate changes, individual unit analysis was used. Units were classified into three populations: excited, inhibited, or unchanged. A non-parametric Wilcoxon signed-rank test was

performed on each unit to determine whether the mean firing rate during the event was significantly different from the baseline. The responses and relative proportions of laser-excited, laser-inhibited, and laser-no-response units before and during the events in males and females were compared using a t-test and chi-square test.

Histology

After experiments, mice were perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was then removed and placed in a 4% paraformaldehyde buffer at 4°C for overnight fixation. After fixation, the brain was cryoprotected in 30% sucrose (wt/vol) at 4°C. Coronal sections were cut at 40 μm on a cryostat (Leica CM1900) for imaging. After rinsing with 0.3% Triton-X 100 (vol/vol) in 0.1 M PBS (30 min) or ice-cold methanol (10 min) and blocking with 10% (wt/vol) normal bovine serum for 1 h at room temperature, sections were incubated (12–24 h at 4°C) with the primary antibodies anti-c-Fos (1:2500, rabbit, Calbiochem), and anti-Esr1 (1:2000, rabbit, Millipore ab64053). After rinsing, sections were incubated with fluorophore-conjugated secondary antibody (1:1000; Millipore) for 2 h at room temperature. The antibodies were diluted in phosphate-buffered saline containing 4% BSA and 0.2% Triton X-100.

To investigate the neuronal activation of VMHvl and its downstream targets for c-Fos quantification after activating the VMHvl^{Esr1} neurons, mice were *I.P.* injected with the CNO to pharmacogenetically activate the VMHvl^{Esr1} neurons in the homecage. These mice were perfused 2 hours later. Sections of the VMHvl, AHN, MPOA, DMH, PVN, vIPAG, pSI, AVPV, dmPAG, and IPAG were cut as described above. To examine the c-Fos expression in the pSI after photostimulation of the projections from VMHvl^{CaMKII} to the pSI, the mice received 10 min laser stimulation and were then perfused 90 min later. The sections of the pSI were collected for anti-c-Fos immunohistochemistry. Nuclei were stained with DAPI, confocal images were captured using 10× and 20× objectives (Olympus FV-1200), and cells were counted manually using Fiji (NIH). Upon completing our behavioral experiments (Figures 1, 2, 3, 4, 5, 6, and 7), we utilized an Olympus VS120 Virtual Slide Microscope to verify viral injection sites in brain sections. Heat maps, manually aligned with the Mouse Paxinos Atlas, were created to depict these sites. For the experiments in Figures 4T–4W, the lack of optimal storage led to diminished fluorescence, preventing the production of accurate heat maps. However, for other specimens, precise heat maps of viral expression were produced as presented in Figures S1–S6.

Fluorescent *in situ* Hybridization

For fluorescent *in situ* hybridization, RNAscope Mm-Slc17a6-C1 (ACDBio), RNAscope Mm-Esr1-C2 probe (ACDBio), RNAscope Mm-Slc17a6-C3 probe (ACDBio), probe (ACDBio) and RNAscope® Multiplex Fluorescent Reagent Kit v2 (ACDBio) were used. Brains from C57 male or female mice were cryosectioned in 30 μm thickness on a cryostat (Leica CM 1950), mounted on slides, heated at 60 °C for 0.5 h, and kept at -80 °C before experiments. All sections were fresh-frozen according to the manufacturer's instructions. On the first day, slices with sections were equilibrated in 1×PBS for 5min. The sections were dehydrated with increasing concentrations of EtOH (50%, 70%, and 100%) for 5 min each time, and then dried for 5 min. Two min after encircling using a hydrophobic barrier pen these were pretreated with hydrogen peroxide for 10 min and washed with PBS for 2 × 2 min. All procedures were performed at room temperature (RT). Sections were then treated with protease III for 30 min at 40 °C in an RNAscope hybridization oven (ACDBio) and washed with PBS for 2 × 2 min. The probe mix contained Slc17a6 (VGLUT2) probes, Esr1 probes, VGAT probes, positive control probes (peptidylprolyl isomerase B, Ppib), or negative control probes [dihydrodipicolinate reductase (dapB) gene]. The sections were hybridized with the pre-warmed probe at 40 °C in the oven for 2h. After hybridization, they were washed with wash buffer for 2 × 2 min at RT. To amplify the hybridization signals, amplifiers (Amp-1, Amp-2, Amp-3) were used in sequence and washed with wash buffer for 2 min at RT. For visualizing the signals, HRP, fluorescence (Alexa Fluor 520 or 570), and HRP blockers from the kit were adapted in turn for 15 min/30 min/15 min at 40 °C, each one requiring washing with wash buffer for 2 × 2 min at RT. Confocal images were captured using 10× and 20× objectives (Olympus FV-1200). Cells were counted manually using Fiji (NIH).

QUANTIFICATION AND STATISTICAL ANALYSIS

No statistical methods were used to pre-determine sample sizes. All data was randomly collected. To assess whether the data values come from a normal distribution, we examined them using the D'Agostino-Pearson omnibus normality test, the Shapiro-Wilk normality test, and the KS normality test. In experiments with paired samples, a paired t-test for the comparison between two groups was used. An RM one-way ANOVA was used for the comparison of three groups for parametric data, and the Wilcoxon matched-pairs signed-rank test was used for the comparison between two groups, or the Friedman test for the comparison of three groups for non-parametric data. In experiments with un-paired samples, an unpaired t-test was used for the comparison between two groups, or an ordinary one-way ANOVA was used for the comparison of three groups for parametric data, and a Mann-Whitney test was used for the comparison between two groups or the Kruskal-Wallis test was used for the comparison of three groups for non-parametric data. In the cases where saturated data are present, survival analysis rather than simple t-tests was used. Multiple comparisons were conducted. Necessary adjustments were made to the data needed for the multiple comparisons. The chi-square test was utilized to compare categorical data, particularly focusing on the proportions of neurons across different groups. All values were calculated with Prism 6.0/8.3 or MATLAB and are presented as the mean ± SEM.