Hormonal gain control of a medial preoptic area social reward circuit

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Neural networks that control reproduction must integrate social and hormonal signals, tune motivation, and coordinate social interactions. However, the neural circuit mechanisms for these processes remain unresolved. The medial preoptic area (mPOA), an essential node for social behaviors, comprises molecularly diverse neurons with widespread projections. Here we identify a steroid-responsive subset of neurotensin (Nts)-expressing mPOA neurons that interface with the ventral tegmental area (VTA) to form a socially engaged reward circuit. Using in vivo two-photon imaging in female mice, we show that mPOA⁵⁵⁵ neurons preferentially encode attractive male cues compared to nonsocial appetitive stimuli. Ovarian hormone signals regulate both the physiological and cue-encoding properties of these cells. Furthermore, optogenetic stimulation of mPOA⁵⁵⁵–VTA circuitry promotes rewarding phenotypes, social approach and striatal dopamine release. Collectively, these data demonstrate that steroid-sensitive mPOA neurons encode ethologically relevant stimuli and co-opt midbrain reward circuits to promote prosocial behaviors critical for species survival.

Results

Identification of a molecularly defined population of steroid-responsive mPOA–VTA neurons

Since diverse cell types have been reported in VTA-projecting mPOA neurons¹²,¹³ and molecular subsets of mPOA populations putatively regulate different types or aspects of social behaviors¹⁴, we first sought...
to identify a molecularly defined population of steroid-responsive mPOA VTA-projecting neurons. The gene encoding the peptide Nts is prominently expressed in the mPOA, and in other hypothalamic regions it has been shown to promote reward-seeking through VTA projections. Injections of retrogradely transported beads into the VTA of female mice resulted in substantial labeling of mPOA neurons, with 96% of Nts-labeled cells colocalizing with VTA beads (Fig. 1a,b). This subpopulation comprised 35% of all mPOA VTA-projecting neurons (Fig. 1a,b). Further, 21% of Nts mPOA neurons expressed mRNA for both estrogen receptor α1 (Esr1) and galanin (Gal) (Fig. 1c–g); Gal-containing mPOA neurons were recently shown to govern parental behaviors in mice. Another 63% contained mRNA for Esr1 but not Gal, and only 3% expressed Gal but not Esr1 (Fig. 1c–g). While the majority of Nts cells expressed Esr1 (84%) (Fig. 1c–g and Supplementary Fig. 1c,d), this appeared to be a subpopulation of all mPOA Esr1 cells.

Previous studies in female rodents indicate that Nts mPOA expression peaks in proestrus and is enhanced by estradiol. We extended these findings to show that 95% of Nts mPOA cells coexpressed the gene for vesicular GABA transporter (Vglut, also known as Slc32a1), and 18% of these also had low to moderate levels of vesicular glutamate transporter (Vglut2, also known as Slc17a6); only 5% expressed Vglut2 alone (Supplementary Fig. 2a–h). These proportions did not vary as a function of the hormonal state; however, administration of estradiol in ovariectomized females increased both mPOA Nts and Vglut mRNA expression intensity compared with that in vehicle-treated controls (Supplementary Fig. 2d–h). Collectively, these data indicate that Nts-containing mPOA neurons comprise a subpopulation of putative GABAergic neurons that project to the VTA, largely express Esr1 steroid receptors, and are responsive to gonadal steroid changes.

**mPOA–VTA** neurons dynamically encode social odor cues

Next we monitored the endogenous activity dynamics of mPOA Nts neurons in response to innately attractive social odor cues in awake female mice using two-photon Ca2+ imaging with single-neuron and cell-type precision. A Cre-inducible virus encoding the genetically encoded fluorescent Ca2+ indicator GCaMP6s (ref. 19) was injected into the mPOA of female mice using two-photon Ca2+ imaging with single-neuron precision (Fig. 1a). Dual Ca2+ imaging and whole-cell patch clamp recordings first established that increasing current injections produced increasing amounts of Ca2+-mediated fluorescence changes in mPOA Nts:GCaMP6s neurons (Supplementary Fig. 4a–c), indicating that GCaMP6s fluorescence correlated with evoked action potentials and can thus serve as a proxy for neural activity. To visualize Ca2+ activity in *vivo*, we gained optical access to this deep brain region through a gradient refractive index (GRIN) lens implanted directly above mPOA Nts::GCaMP6s neurons and attached a head ring for subsequent two-photon imaging (Fig. 2a and Supplementary Fig. 4d–f). Following recovery and time to permit GCaMP6s expression, mPOA Nts:GCaMP6s neurons from female mice in proestrus were imaged during multiple time-locked, automated trials wherein vaporized social odors were presented (Fig. 2a). Intact male urine served as an attractive social cue with reproductive relevance and ovariectomized female urine served as a conspecific urine control; both odor types were innately attractive to female mice (Supplementary Fig. 5a–g). Ca2+ dynamics were reliably detected in individual mPOA Nts:GCaMP6s cells with single-neuron precision (Fig. 2b,c, Supplementary Fig. 4d–f and Supplementary Video 1), and we identified a subset of individual mPOA Nts neurons that were preferentially excited by male odor (Fig. 2b–d and Supplementary Video 2). Across all animals tested, a greater proportion of mPOA Nts:GCaMP6s cells were excited by male odor (37%) than by female odor (8%) (Fig. 2d). Moreover, a direct odor-type comparison of cells that were significantly excited by male odor revealed that male-excited cells were less responsive to female odor (Fig. 2e). These data indicate that a large proportion of mPOA Nts neurons dynamically encode social odor cues, with subsets preferentially responding to reproducitively relevant male signals.

**mPOA** neurons encode reproductive male cues in a steroid-gated manner

Gonadal steroids, including estradiol, can enhance the processing of reproductive social signals, promote mate attraction and differentially regulate mPOA neuronal activity in *vivo* (which could reflect differences in cell types or steroid receptor expression). We next evaluated whether ovarian steroids influence social cue-encoding dynamics by tracking odor-evoked Ca2+ activity from the same mPOA Nts:GCaMP6s neurons over the days before and after steroid priming in ovariectomized females (Fig. 3a). In a separate cohort of ovariectomized females, steroid priming of estradiol approximately 5 h before testing enhanced behavioral attraction to male urine, compared with vehicle treatment. This behavioral response was sustained following a second injection of progesterone (a steroid regimen known to induce sexual receptivity) (Fig. 3a,b and Supplementary Fig. 6a–e). Using this priming schedule for imaging, we detected Ca2+...
Trials data indicate that mPOA Nts neurons adjust their male odor encoding after estradiol replacement (Fig. 3), but not following oil odor (after home cage exposure) (Fig. 3a,b). Individual neurons were excited by male urine and/or female urine, but these cells were largely unresponsive to or inhibited by peanut oil (Fig. 3a-c). Further, a greater proportion of neurons were excited by male urine odor (28%) compared to intact female urine odor (8%) or peanut oil odor (10%) (Fig. 3d). These single-neuron comparisons demonstrate that distinct forms of attractive stimuli can recruit largely non-overlapping mPOA Nts neuron ensembles and that social versus nonsocial appetitive cue encoding is dissociable at the individual-neuron level.

Steroids enhance neuronal excitability in mPOA Nts neurons

Ovarian steroids altered not only the odor-encoding dynamics (Fig. 3), but also the basal and physiological properties of mPOA Nts neurons. Imaging spontaneous Ca2+ activity dynamics in intact cycling females across 2 d of the estrous cycle revealed that individual mPOA Nts neurons adjusted their spontaneous activity dynamics across hormonal states; specifically, they showed enhanced Ca2+ activity on the afternoon of proestrus (when estradiol levels peak) compared to the afternoon of estrus (Supplementary Fig. 6i–l) and following estradiol-priming in ovariecotomized females compared with vehicle (Fig. 5a–d). To explore potential underlying physiological mechanisms, we performed ex vivo patch-clamp and fluorescence recordings from mPOA Nts neurons. We found that estradiol priming increased the number of evoked action potentials (Fig. 5e,f) and
resulted in larger Ca²⁺ signals (Supplementary Fig. 4a–c), compared with those in controls. mPOA NST neurons also showed reduced action potential half-width and an increase in A-type K⁺ channel conductance following estradiol replacement (Fig. 5g,h). This suggests that estradiol controls mPOA NST excitability at least in part by increasing the function of delayed rectifier K⁺ channels, which rapidly repolarize the neurons to allow high-frequency action potential generation. Collectively, these data illustrate how estradiol enhances the intrinsic excitability of mPOA NST neurons and might permit enhanced sensitivity to appetitive cues.

Stimulation of mPOA NST–VTA neurons promotes reward and ventral striatal dopamine release in a steroid-responsive manner

Appetitive cues excited mPOA NST neurons, and we next tested whether optogenetic activation of these neurons would convey positive valence signals and reinforce behavioral actions. We targeted mPOA NST neurons with the light-gated cation channel channelrhodopsin-2 (ChR2) fused to eYFP, and implanted optical fibers above the mPOA to permit photostimulation of mPOA NST–VTA fibers. mPOA NST::ChR2 neurons from two adult male mice were confirmed to be the target of mPOA NST cells (Supplementary Fig. 7a,b). In contrast to females, photostimulation produced a more consistent real-time place preference across 4 consecutive days in mPOA NST::ChR2 males, compared with mPOA NST::eYFP controls (Fig. 6i,j). In addition, photostimulation enhanced movement velocity in both males and females (Supplementary Fig. 7d,e). We confirmed that this approach resulted in the activation of mPOA NST neurons, as photostimulation induced Fos expression in mPOA NST::ChR2 mice but not mPOA NST::eYFP controls (Supplementary Fig. 8a–c). Collectively, these data demonstrate that stimulation of mPOA NST neurons produces reinforcement in both sexes.

Although mPOA NST neurons promoted reward-related phenotypes that varied in magnitude across the estrous cycle, it remained unclear whether steroids directly modulate these effects and how these cells orchestrate the output of motivated behavior through recruitment of downstream circuit targets. mPOA NST::ChR2-assisted anterograde neuroanatomical tracing revealed dense fiber expression in the VTA from these cells (Fig. 7a), consistent with the identification of mPOA NST cells as VTA-projecting neurons (Fig. 1a,b). We then targeted optogenetic stimulation to either the mPOA NST cell bodies or mPOA NST–VTA fibers before and after steroid priming (estradiol) in ovariectomized female mice (Fig. 7a). Photostimulation of mPOA NST cell bodies or mPOA NST–VTA fibers induced a modest real-time place preference in mice that was substantially amplified following estradiol replacement, but not after repeated stimulation under vehicle conditions (Fig. 7c and Supplementary Fig. 9a).
Figure 4 mPOA\textsuperscript{Nts} encoding comparison of social and nonsocial odor cues. (a) Spatial map of cell outlines in response to distinct odor cues. Mask displays binary odor response of cells excited by male odor (blue), female odor (pink), both male and female odor (yellow), nonsocial peanut oil odor (orange), all odors (purple), or none of the odors (white). (b) Example Ca\textsuperscript{2+} trace from 3 neurons indicated by arrows in the spatial map. Traces were averaged across 7 odor trials for male urine, female urine or peanut oil. (c) Each row of the heat plot depicts the Ca\textsuperscript{2+} response of an individual mPOA\textsuperscript{Nts}:GCaMP6 neuron averaged across male (left plot), female (middle plot) or peanut (right) odor trials. Heat plots are indexed in the same order for comparison and cells were sorted by male odor response. Vertical axis: normalized Ca\textsuperscript{2+} fluorescence (FI/F\textsubscript{0}). Scale bar, 10 s (n = 155 cells combined across 3 mice). (d) Pie charts show the percentage of mPOA\textsuperscript{Nts}:GCaMP6 cells significantly excited or inhibited by male, female or peanut odors (Wilcoxon T-test, all P < 0.05, n = 155 cells combined across 3 mice).

Notably, this steroid priming regimen of estradiol followed by progesterone, known to induce sexual receptivity\textsuperscript{32}, resulted in a sustained increase in the reward effects of mPOA\textsuperscript{Nts}::ChR2 stimulation for many consecutive weeks (Supplementary Fig. 9a).

To determine whether activation of the mPOA\textsuperscript{Nts}–VTA pathway evoked mesolimbic dopamine release, we employed \textit{in vivo} fast-scan cyclic voltammetry in anesthetized females and recorded dopamine release in the NAc (Fig. 7c). Photostimulation of mPOA\textsuperscript{Nts}–VTA fibers evoked dopamine release in the NAc that was greater in estradiol-primed females compared with controls (Fig. 7c–e). Together, these data indicate that mPOA\textsuperscript{Nts} neurons promote rewarding phenotypes through interactions with mesoliminc dopaminergic circuits in the VTA. Further, these behavioral outputs as well as their physiological dopamine correlations are magnified in steroid-primed females, functionally identifying a molecularly defined steroid-responsive reward pathway.

Optogenetic modulation of mPOA\textsuperscript{Nts} neurons regulates social attraction

We next examined whether mPOA\textsuperscript{Nts} neurons and their projections to the VTA are involved in social approach behavior toward a reproductive social stimulus. We measured attraction and preference for an intact male versus ovariectomized female stimulus in a social investigation assay, during alternating time epochs of photostimulation (Online Methods and Fig. 8a). Time epochs were selected over proximity- or zone-related photostimulation parameters, since activation of this circuit reinforces behavior and could condition responses that might not be socially driven. Testing was conducted in the same females and males used as above (Supplementary Fig. 9a–d). Photostimulation of mPOA\textsuperscript{Nts}::ChR2 neurons or the mPOA–VTA\textsuperscript{Nts}::ChR2 pathway enhanced male preference and increased the amount of time spent in the male social interaction zone only in ChR2-expressing females previously treated with steroids (Fig. 8b–e and Supplementary Video 5). In males, photostimulation of mPOA\textsuperscript{Nts}::ChR2 enhanced female preference and increased the amount of time spent in the female social interaction zone (Supplementary Fig. 9b–d). Together, these data suggest that mPOA\textsuperscript{Nts} neurons direct behavior toward opposite-sex conspecifics in both sexes to drive social attraction toward a potential mate.
Since steroids act to promote mate attraction in females, we next tested whether endogenous activity of mPOA<sup>Nts</sup> neurons is required for sex-specific approach behavior in the presence of steroid priming. mPOA<sup>Nts</sup> neurons were targeted to express the inhibitory light-gated chloride pump halorhodopsin (NpHR) or control (eYFP) in orexinergic female mice. Steroid priming (estradiol and progesterone) plus mating was necessary to first naturally elicit male preference and reduced male but not female investigation (Fig. 8f–h and Supplementary Fig. 9f). Since mate approach is facilitated by hormones and chemosensory cues, we also tested male odor preference in steroid-primed females (Fig. 8i) and found that photo inhibition of mPOA<sup>Nts</sup>:NpHR females impaired male preference and reduced male but not female social investigation, since photoinhibition did not produce a real-time place aversion in the presence or absence of steroid priming (Supplementary Fig. 9g). Finally, optogenetic modulation of mPOA<sup>Nts</sup> neurons did not affect palatable food consumption, although mPOA<sup>Nts</sup>:ChR2 stimulation increased food zone approach bouts (Supplementary Fig. 10a–o). This is in line with our in vivo imaging data, indicating that some mPOA<sup>Nts</sup> neurons are excited by food-related odors rather than male or female social odors (Fig. 4). Together, these experiments indicate that steroids enhance mPOA<sup>Nts</sup> neuronal function to facilitate approach behavior toward attractive social and nonsocial stimuli.

**DISCUSSION**

Historically, the mPOA has been considered an evolutionarily conserved region for social behaviors and homeostatic functions. However, it has remained unclear whether mPOA circuitry orchestrates social behaviors in both sexes. Together, these experiments indicate that steroids enhance mPOA<sup>Nts</sup> neuronal function to facilitate approach behavior toward attractive social and nonsocial stimuli.
The appetitive and reinforcing aspects of social and nonsocial motivated behavior. Here we provide evidence that mPOA–VTA<sup>Nts</sup> neurons convey positive valence signals, reinforce behavioral actions, and regulate ventral striatal dopamine release. It seems likely that mPOA–VTA<sup>Nts</sup> fibers directly inhibit VTA GABAergic neurons to evoke dopamine release and promote reward-related behaviors<sup>24,35</sup>. Thus, we speculate that activation of mPOA–VTA<sup>Nts</sup> fibers produces ventral striatal dopamine release via disinhibition of VTA dopamine neurons. Consistent with this, GABAergic VTA inputs, such as those from the lateral hypothalamus and bed nucleus of the stria terminalis, largely act to inhibit VTA GABA neurons to evoke dopamine release and promote reward-related behaviors<sup>24,35</sup>.

While we found that most mPOA<sup>Nts</sup> neurons project to the VTA, this comprised roughly a third of all mPOA–VTA projecting neurons (though viral targeting and retrograde tracing does not label every Nts or VTA projecting neuron). This suggests that other molecularly defined mPOA subsets may have different encoding and behavioral properties from those described here. In the neighboring ventral bed nucleus of the stria terminals, GABAergic and glutamatergic VTA-projecting neurons differentially control reward and aversive motivational states, respectively<sup>34</sup>. Here, mPOA–VTA<sup>Nts</sup> stimulation promoted not only rewarding phenotypes, but also approach behavior toward an opposite-sex conspecific. Molecularly defined VTA-projection neurons might control distinct types of motivated behaviors (for example, social, feeding, aversion), as well as different aspects of those behaviors (for example, appetitive versus consummatory). Using in vivo Ca<sup>2+</sup> imaging, we found that some mPOA<sup>Nts</sup> neurons were engaged by multiple types of social stimuli but were unresponsive to appetitive nonsocial odor, and vice versa. This suggests that social versus nonsocial appetitive stimuli might recruit different neural ensembles within this mPOA reward circuit, and it is largely unclear whether different types of rewards are processed through distinct pathways within reward systems. In addition to distinctions in which neurons were excited, we observed variance in the magnitude of excitation in response to different odors within the same neurons. Different types of appetitive stimuli produced varying degrees of mPOA–VTA<sup>Nts</sup> circuit activation, with opposite-sex stimuli being the most potent activator, which might serve to promote and reinforce reproductive behaviors essential for species survival.

While male odor was the most robust modulator of mPOA<sup>Nts</sup> neural activity, it is unknown whether this circuit is biased toward reproductive stimuli restricted to mating or if this extends to other forms of social stimuli relevant to offspring and species survival, such as infant cues. A subset of mPOA<sup>Nts</sup> neurons coexpressed Gal, which has previously served as a marker for mPOA neurons essential for parental behaviors in both male and female mice<sup>14</sup>. Maternal functions also require mPOA–VTA circuits<sup>4,6</sup>, but it is unknown whether the motivational aspects of mating and parenting share a common mPOA–VTA pathway that is appropriately tuned by hormonal profiles and social or reproductive experience or whether there are distinct pathways for different social behaviors. Steroids including estrogen influence peptide hormones<sup>4</sup>, which influence mating and infant care through actions in the mPOA, putatively on VTA-projecting neurons<sup>4,6</sup>. Thus hormones, peptides and experience-induced changes likely modify and tune this pathway in a context-specific manner. Mating and parenting induce distinct mPOA Fos activation patterns in male mice<sup>14</sup>, which could reflect the diversity of sensory and motoric aspects associated with different types of social interactions.

Here we restricted our imaging to different types of social and nonsocial odors to rigorously differentiate between distinct salient stimuli processed through the same sensory modality. While less naturalistic, we used automated time-locked odor delivery in head-fixed mice to avoid movement interference, control the proximity and timing of the odor stimulus (rather than the mouse controlling it), and allow multiple trials to ensure consistency. Since females were more innately attracted to male odor stimuli over female stimuli and male odor presentation produced the greatest activation of mPOA<sup>Nts</sup> neurons, this could reflect a bias toward the most motivating stimulus and/or relate to a male-specific sensory detection. In the present optogenetic tests, we also detected an opposite-sex behavioral preference, although it is possible that stimulation of this pathway might drive social approach toward other forms of social or same-sex stimuli in the absence of a competing potential mate. It also remains unknown which sensory or chemical components associated with detecting male odor were driving activation of this pathway. Compared with female urine, intact male urine contains a different profile of gonadal steroids.
steroids and male-specific pheromone proteins, including major urinary proteins, such as darcin, that promote male attraction in female rodents. Future studies should use in vivo chronic imaging approaches from molecular and projection-specific mPOA populations under a range of social and hormonal sex-specific contexts and following social experience.

The incentive value of particular stimulus is dependent on a number of internal physiological factors (for example, hormonal levels and the homeostatic state of the animal) that are in constant flux. Here mPOA neurons adjusted their basal and evoked-odor activity dynamics across hormonal states. Multiple chemosensory inputs converge in the mPOA, and our studies indicate that steroids prime social odor-sensing neurons to be more responsive to male signals. Estrogens can induce slower genomic actions through binding of nuclear steroid receptors that act as transcription factors to promote or repress gene expression in the hypothalamus. Here, estradiol enhanced Ns and Vgl mRNA in mPOA neurons, though since the Ns promoter lacks palindromic estrogen response elements, estradiol likely increased mPOA Ns expression through a PKA-dependent signaling pathway, as others have found. Estradiol can also exert rapid nongenomic actions by acting at membrane-bound receptors or ion channels that stimulate intracellular signaling pathways and can increase or decrease cell excitability. Indeed, we showed that estradiol enhanced the intrinsic excitability of mPOA Ns neurons in part by increasing delayed rectifier K⁺ channel conductance, thus preventing action potential attenuation and failure. In agreement with our slice data, estradiol priming before imaging enhanced Ca²⁺ dynamics, which were characterized by elevated event amplitude and duration. Since steroids were administered hours and days before preparing tissue for slice electrophysiological recordings or examining behavioral and neuronal encoding, this time frame permits slower genomic actions that alter the expression of genes, receptors or ion channels. It is also possible that prior in vivo estradiol altered the expression of K⁺ channels, as seen in the arcuate nucleus, and/or that estradiol in the bath, for electrophysiological recordings, directly affected K⁺ channel activity.
Apart from estradiol, a number of other steroid and peptide hormones could also orchestrate the activity of this circuit in males and females. Here, estradiol was sufficient to enhance responsiveness to male odor cues and amplify the rewarding effects of mPOA–VTA

brief introduction to the use of circuit strategies in conjunction with in vivo neural encoding properties and circuit functions modulated by different hormonal contexts. These studies suggest that neural networks excited by social signals are embedded within valence-processing circuits and can directly orchestrate motivational states. While many studies have used in vivo circuit strategies to study social or motivated behaviors, our understanding of how positive and negative valence circuits are intertwined with social networks is in its infancy. Future studies can use circuit strategies in conjunction with in vivo imaging to resolve whether precise neurons encode with reward-type specificity and whether the same networks integrate not only different types of rewarding stimuli, but also aversive signals that could underlie complex interactions between stress, social functioning and reward-related deficits. Collectively, these studies demonstrate how hormonal signals fine-tune neural circuit dynamics at key reproductive network nodes to translate sensory input into socially directed motivated behavior essential for species survival. Uncovering neural circuits that function to bridge social and reward processing will provide important insights for social and affective disorders. Moreover, while steroid-mediated shifts in neural processing are generally adaptive for reproductive strategies, such actions can destabilize mood in some women and contribute to hormonal and sex-related influences in drug addiction. Further elucidation of steroid-sensitive circuits that control motivational states may provide new therapeutic targets for sex-biased psychiatric and reproductive mood disorders.

Conclusions

We implemented a multifaceted approach to disentangle in vivo neural encoding properties and circuit functions modulated by different hormonal contexts. These studies suggest that neural networks excited by social signals are embedded within valence-processing circuits and can directly orchestrate motivational states. While many studies have used in vivo circuit strategies to study social or motivated behaviors, our understanding of how positive and negative valence circuits are intertwined with social networks is in its infancy. Future studies can use circuit strategies in conjunction with in vivo imaging to resolve whether precise neurons encode with reward-type specificity and whether the same networks integrate not only different types of rewarding stimuli, but also aversive signals that could underlie complex interactions between stress, social functioning and reward-related deficits.

Collectively, these studies demonstrate how hormonal signals fine-tune neural circuit dynamics at key reproductive network nodes to translate sensory input into socially directed motivated behavior essential for species survival. Uncovering neural circuits that function to bridge social and reward processing will provide important insights for social and affective disorders. Moreover, while steroid-mediated shifts in neural processing are generally adaptive for reproductive strategies, such actions can destabilize mood in some women and contribute to hormonal and sex-related influences in drug addiction. Further elucidation of steroid-sensitive circuits that control motivational states may provide new therapeutic targets for sex-biased psychiatric and reproductive mood disorders.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.A.M. and G.D.S. designed the experiments and wrote the manuscript. J.A.M. provided input from all authors. J.E.R. and J.E.R. performed physiology recordings. E.A.B. performed fast-scan cyclic voltammetry. M.A.R. wrote code for in vivo imaging data analysis. O.K. and Z.A.M. performed in situ hybridization. N.W.M. assisted with tissue processing. J.A.M. performed all surgeries, behavioral assays, two-photon and confocal imaging experiments. D.R.R. and G.D.S. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals and surgeries. Subjects. Nts-IRES-Cre mice20 were maintained and Nts-Cre or wild type littermate mice were group housed according to sex (20–30 g, 6–9 weeks old) until surgery or behavioral testing. Mice were maintained on a reverse 12-h light cycle (lights off at 07:00) with ad libitum access to food and water. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted by the NIH, and with approval of the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill (UNC).

Subject history. All wild-type animals were randomly assigned to groups. In cases2 of experiments with transgenic mice, groups requiring the presence or absence of Cre were determined by the genotype of the mouse and then randomly distributed. Animals were naïve to experimental testing before the beginning of a study. Multiple optogenetic experiments were conducted in the same animal subjects for the following cohorts: intact females for optogenetic activation were tested across the estrous cycle for the real-time place preference and optical self-stimulation; intact males for optogenetic activation were tested for real-time place preference and social approach behavior; ovariectomized females for optogenetic activation were tested for real-time place preference, social approach assay and high-fat diet assay; and ovariectomized males for optogenetic inhibition were tested for real-time place preference, social approach assay, high-fat diet assay and odor preference.

General procedures. For all surgeries described below, mice were anesthetized with 0.8–1.5% isoflurane mixed with pure oxygen (1 L/min) and placed in a stereotactic frame (David Kopf Instruments), and all surgical instruments and materials were sterilized. Ophthalmic ointment (Akorn) and a topical anesthetic (2% Lidocaine; Akorn) were applied during surgeries, and subcutaneous injections of sterile saline (0.9% NaCl in water) were administered to prevent dehydration. Following surgeries, mice received acetaminophen in their drinking water for 2 d and were monitored for 7 d or until full recovery.

Viral constructs. Cre-inducible AAV5-EF1-DIO-hChR2(H134R)-eYFP (4.3 × 1012 infectious units/mL), AAV5-EF1-DIO-eYFP (6.0 × 1012 infectious units/mL), AAV5-EF1-DIO-eNpHR3.0-eYFP (6.0 × 1012 infectious units/mL) and AAVδg-EF1-DIO-GCaMP6s (3.1 × 1012 infectious units/mL) were packaged by the UNC vector core and delivered stereotaxically in the mPOA of anesthetized mice.

Viral and optogenetic surgery. For all virus surgeries for optogenetics, slice physiology or anatomical tracing, animals were anesthetized and treated as described above. Bilateral injections of Cre-inducible virus (500 nl/side) were administered at a 10° angle into the mPOA (relative to bregma: AP, +0.15 mm; ML, ± 1.30 mm; DV, −5.10 mm). For retrograde anatomical tracing, red Retrobeads (Lumafluor) were bilaterally injected (300 nl/side) at a 10° angle into the VTA (relative to bregma: AP, −3.12 mm; ML, ± 1.30 mm; DV, −5.0 mm). For optogenetic surgeries, custom-made chronic optical fibers39 were also implanted bilaterally at a 10° angle ~0.50 mm above the mPOA or VTA target site relative to the coordinates listed above. For behavioral experiments targeting optogenetic stimulation to the mPOA or VTA, the same mice were used and fibers were implanted in both regions. Following surgery, we allowed sufficient time for viral infection and transgene expression. The time course between viral injections and the start of manipulations during a social preference assay, subjects were placed in a standard slice physiology and 7–8 weeks for optogenetic terminal manipulations.

GRIN lens surgery. For deep-brain in vivo two-photon Ca2+ imaging experiments, animals were anesthetized as described above and the Cre-inducible genetically encoded Ca2+ indicator GCaMP6s (ref. 19) was unilaterally injected into the mPOA (500 nl at two–D–V axis positions; relative to bregma AP, +0.05 mm; ML, ± 0.25 mm; DV, −4.95 and −5.15 mm). Prior to injections, the craniotomy site was carefully prepared and tissue aspirated with minimal bleeding41. Then a gradient refractive index (GRIN) lens (7.3 mm length, 0.6 mm diameter, Inscopix GLP-0673) was slowly stereotaxically lowered to a location directly above the mPOA at different coordinates than the viral injection site (relative to bregma AP, +0.10 mm; ML, ± 0.35 mm; DV, −4.75 mm) to avoid placing the lens in the immediate area of the injection track, which typically exhibits autofluorescence that could interfere with imaging. Lastly, a custom-made ring (stainless steel; 5 mm ID, 11 mm OD) was attached to the dental cement during surgery to allow subsequent head fixation (see Fig. 5a). Animals were monitored for GCaMP6 expression 4 weeks after surgery using two-photon microscopy as described below, and only those with dynamic GCaMP6 expression in at least 50 cells and absent of autofluorescence were used for imaging experiments.

Ovariectomy and hormone replacement. Females were anesthetized as described above and ovariectomized through bilateral flank incisions. The ovary was located in a fat pad just beneath the muscles and, using forceps, the periovarian fat was gently grasped to lift and exteriorize the ovary. The uterine horn was then returned into the abdomen and the process repeated on the other side. The muscle of the posterior abdominal wall was then sutured and the exterior skin incision closed with sterile surgical staples. Mice were monitored daily following surgery and staples were removed a week later. Females recovered for at least 10 d before testing. Estradiol was administered at 10 µg and progesterone at 500 µg in 0.05 ml of sesame oil delivery s.c. and 48 h apart since this regimen, referred to as steroid priming, has been shown to induce sexual receptivity and these levels of estradiol are comparable to physiological proestrus peak levels52,53. Vehicle injections consisted of 0.05 ml of sesame oil. Behavioral testing or physiological recordings were all conducted approximately 4–5 h after the injection.

Estrous cycle monitoring. Female mice are spontaneous ovulators and typically have a 4–6 day estrous cycle that consists of four stages: proestrus, estrus, diestrus I/metestrus and diestrus II. Mice were habituated to handling and vaginal smears before behavioral experiments. Before 09:00 h, mice were swabbed daily regardless of behavioral testing to check for consistent cycling52,53 and underwent behavioral testing in the afternoon between 1300 and 1700 h. Using a disposable pipette, a small volume of physiological sterile saline was placed near the opening of the vagina and 100 µl of saline was flushed in four repetitions without insertion to avoid pseudopregnancy. The smear was displaced onto a glass slide and immediately examined under a brightfield microscope at 20×. The identification of estrous stages was based on characteristic cell type appearance observed and the density of each cell type in the vaginal secretion. Estrous stage was identified by changes in cell appearance across the cycle that reflects circulating gonadal steroids. Note that “estrus” does not refer to “behavioral estrus,” or the state of being sexually receptive. Females were only tested for behavior on days when a clear smear was visible and the cycle stage could easily be identified. In cases where a smear was unclear or an animal did not resume the anticipated estrous stage following optogenetic or behavioral stimulation, the animal was not tested until resuming consistent estrous cycling.

Behavioral optogenetics. General procedures for light delivery. Prior to behavioral testing, animals were habituated to handling and patch cable tethering in their home cage for at least four habituation sessions lasting ~10 min. For optogenetic manipulations, light ~10 mW from diode-pumped solid-state lasers (473 nm or 532 nm) was delivered through custom-made patch cables attached to the implanted chronic fibers on the animal’s head as described previously34,50. For photoactivation, 473-nm light was pulsed at 20 Hz with a pulse width of 5 ms. For photoinhibition experiments, 532-nm light was delivered constantly. Animals were able to freely move within the testing chambers with little hindrance, and all behavioral testing was conducted under red incandescent lighting between 13:00 and 18:00 h. For each behavioral assay, Ethovision or MedPC software and Arduino microcontrollers were used to control light delivery parameters through a TTL pulse.

Behavioral testing: reward and feeding assays. For real-time place preference testing, subjects controlled the amount of light stimulation as determined by their center point location within the testing area in 20-min sessions. For optical self-stimulation, animals were given access to an active and inactive port and could nose poke to receive optical stimulation over a 30-min test period. For additional details on these behavioral assays, see ref. 34. For optogenetic manipulation during a free-feeding assay, animals were given access to a high-fat food and consumption measured as previously described54,55.

Behavioral testing: social and odor preference assays. For optogenetic manipulations during a social preference assay, subjects were placed in a standard
three-chamber social choice arena57, but instead two social stimuli were used (see Fig. 8a). Stimulus mice were previously habituated to the holding chambers for at least 3 d before experimental testing. For female test subjects a wild-type intact adult male and ovariectomized female stimulus mouse was placed into the respective social interaction holding chambers. For male test subjects, a hormone-primed ovariectomized female and adult male were used. Subjects were first habituated to the area in the absence of light delivery or social stimuli and screened for any innate side preference to the area. Light delivery occurred in alternating 5-min epochs for a total of 20 min. For NpHR cohorts, hormone-primed females were first mated in their home cage to induce a male preference before behavioral testing. Since we observed positive reinforcing properties in response mPOANs stimulation, we used optogenetic modulation parameters that were not time-locked to occur with social proximity, since pairing could artificially condition and reinforce social approach behavior.

For odor preference testing, subjects were placed into a rectangular arena and testing lasted for 20 min, with light delivery alternated in 5-min epochs. Subjects were first habituated to the area for 10 min and screened for any innate side preference. Then two square blocks placed on opposite walls of the arena tightly held a cotton swab containing either urine or physiological saline (60 µL per swab; Fig. 8i). Wild-type stimulus mice were habituated to a chamber containing a wire mesh floor without bedding and a drip pan for the collection of fresh mouse urine. Urine was pooled from four group-housed male mice and one singly housed adult male, since male urine contains female-attracting proteins (for example, darcin56) that are variable in expression across mice and can be influenced by social environment58. Female urine was pooled from five ovariectomized group-housed females. Peanut oil was used at full strength and 60 µL per swab during testing. Peanut oil was first applied to a piece of food in the home cage the day before behavioral testing or imaging odor-evoked responses.

Behavioral analysis. All behavioral experiments were recorded live with a video camera and PC equipped with live tracking software (Noldus, Ethovision XT). Automated measures of movement (velocity and distance) and the frequency and duration of time spent in each designated zone of the area was generated using live tracking Ethovision software and calculated with respect to light condition. Preference indices for social or food stimuli were computed according to these metrics as previously described59. Food consumption and direct food contact were manually scored with assisted software (Noldus, Observer). Since two subject males climbed out of the area or around the roof of the area during the majority of this test, these mice were excluded from data analysis.

Two-photon in vivo calcium imaging. Head-fixed imaging. To visualize Ca2+ dynamics in vivo, GCaMP6s was expressed in mPOANs neurons and a GRIN lens implanted to gain optical access to this deep brain region as described above (see “GRIN lens surgery”). Mice were habituated to head fixation for at least 4 non-consecutive days with increasing time intervals (5–30 min) and then two-photon microscopy was used to visualize activity dynamics of mPOANs neurons in vivo. The last habituation session occurred in conjunction with two-photon imaging to select a particular field of view (FOV) by adjusting the imaging plane (z axis) to select a different group of cells across multiple FOVs within each subject. Multiple FOVs were collected for the baseline spontaneous imaging sessions and a single FOV with the most cells was chosen for stimulus odor-evoked imaging sessions (to avoid time confounds).

Imaging: schedule relative to hormone contexts. Females were not imaged more than twice a week to avoid disruption of the natural cycle. Females were smeared in the morning (see “Estrous cycle monitoring”) and imaging was conducted in the afternoon as described above. For all hormone manipulation experiments in ovariectomized females, mice were imaged ~4–5 h after injections as described above (for more hormone details, see “Ovariectomy and hormone replacement”).

Spontaneous Ca2+ activity. To examine spontaneous Ca2+ events across hormonal states, females were smeared (intact females) or injected (ovariectomized females) before 09:00 h and mPOANs::GCaMP6s cells imaged in vivo ~4 h later. Baseline scans from each FOV were acquired for 10 min at 5 Hz as described below.

Odor-evoked Ca2+ activity. For odorant delivery, fresh urine was collected using the same 5 male or female animals that were group-housed and urine was pooled (see “Behavioral optogenetic methods”), since there are individual differences in the amount of proteins in male urine that attract the female mice. Urine was collected from the same group of stimulus mice each day and used across all imaging studies to ensure consistency within and between mice. Pooled mouse urine from each sex or peanut oil was added to a chamber, vaporized through an airflow tank containing air similar to room air and flowed out through a mask placed in front of but not touching the animal’s snout, with a vacuum line on the adjacent side to remove any residual odor. Specifically, 160 µL of urine diluted in dipropylene glycol was placed into a small chamber and odorants were delivered using a custom-made ofaomcter. Medical air (2 L/min) served as positive pressure that led to a mask situated in front of the subject’s snout with a vacuum line to help remove any residual odor (see Fig. 2a). To time odorant delivery during imaging, a microcontroller was programmed to initiate imaging and trigger a solenoid that switched from air to the odor tank. During each imaging session, two-photon scanning was triggered for each trial 10 s before odorant delivery, and a 40-s video was collected for each trial. Each odorant trial was delivered in 6 or 7 repetitions with a 2- to 3-min inter-trial interval to avoid odor habituation. The number of trials is specified in the figures and associated legends.

Two-photon imaging acquisition. A two-photon Olympus microscope (FVMPRS) was equipped with the following: a hybrid scanning core set with galvanometers and fast resonant scanners (allowing up to 30 Hz frame-rate acquisition; set to 5 Hz); GaAsP PMT photo detectors with adjustable voltage, gain, and offset; a long working distance 20× air objective designed for optical transmission at infrared and visible wavelengths (Olympus, LCPN2XIR, 0.45 NA, 8.3 mm WD); a software-controlled modular stage located on a manual z deck; and a tunable Mai-Tai Deep See laser with dispersion compensation (Spectra Physics, laser set to 955 nm, ~100 fs pulse width) with automated four-axis alignment. Data were both acquired and processed using a computer equipped with FluoView (Olympus, FV1200) software. For all sessions, time series images of mPOANs::GCaMP6s cells were collected using the resonant scanner at 30 Hz with six-frame on-the-fly frame averaging, which permits final image acquisition at 5 Hz. Software acquisition settings were optimized for each subject and set within the following range: laser intensity: less than ~50 mW, PMT voltage: 650–675, gain: 1.5, offset: 2, scan size: 512 × 512, zoom 1–1.5, aspect ratio: 1:1. For chronic imaging experiments the same FOV was acquired under the same imaging parameters and software acquisition settings across all days for comparison.

Data extraction and analysis. Following acquisition, data were exported as a tiff series and motion-corrected using a planar hidden Markov model (SIMA v1.3; ref. 59), and regions of interest (ROIs) were hand drawn around each cell or dendrite on the s.d. projection tiff image of the motion-corrected video using ImageJ. For chronic imaging experiments, the same ROI series file was loaded onto the tiff image and individual ROIs were adjusted to ensure proper alignment with each cell; only cells that could be reliably identified at the same FOV were included in subsequent analysis. Next, normalized calcium signals were extracted from the ROIs using the SIMA objects and motion-corrected imaging video, and all subsequent analyses were conducted using freely available Python packages (SIMA, numpy, scipy, pandas, seaborn). All analysis code can be found at http://www.gitub.com/stuberlab/.

Event detection. For each ROI, extracted fluorescence intensity time series data were independently z-scored and then smoothed with a three-frame rolling mean. Using the normalized and smoothed time series data, calcium transients were defined as events if the transient magnitude exceeded 1 s.d. for at least five frames (1 s). The start and end of each event were defined as the points at which the normalized signal crossed the 1 s.d. threshold, and the duration of each event was calculated using these boundaries. The peak amplitude was the maximum normalized intensity value within each event. For rare instances in which acquisition started or ended during an ongoing calcium event, that event was removed from all subsequent analyses.

Detecting odor-evoked responses. Analyses were performed on SIMA-corrected fluorescence intensity time series data. For each ROI and each trial, normalized intensity values were calculated by dividing each value by the mean intensity of the baseline epoch (10 s immediately preceding odor presentation). Cell responsivity was assessed for each cell using the Wilcoxon signed-rank test across 6 or 7 trials per session. Mean baseline fluorescence was compared to the mean fluorescence during the odor epoch. Excited ROIs were those in which the odor response was significantly (P < 0.05) greater than baseline, and inhibited ROIs were those in which the odor response was significantly (P < 0.05) lower than baseline. The number of trials was consistent for each experiment (Fig. 2: 6 trials, Figs. 3 and 4: 7 trials), and all odor trials were included in the analysis for all subjects and all ROIs.
In vivo fast-scan cyclic voltammetry. Fast-scan cyclic voltammetry (FSCV) experiments were conducted using method described in previous studies. Briefly, mice were anesthetized and placed in a stereotaxic frame. A craniotomy was done above the nucleus accumbens (AP, +1.0 mm; ML, 1.0 mm) and the VTA (AP, −3.1 mm; ML, 0.3 mm). An Ag/AgCl reference electrode was implanted in the contralateral forebrain. An optical fiber (200 µm) was placed for the activation of ChR2-expressing VTA neurons (DV, from −4.0 to −4.6 mm). A carbon fiber electrode (−100 µm in length, 6 µm diameter) for voltammetric recordings was then lowered into the nucleus accumbens (DV, from −4.0 to −4.6 mm) in 0.2 mm intervals. Voltammetric measurements were made every 100 ms by application of a triangular waveform (−0.4 V to +1.3 V to −0.4 V versus Ag/AgCl at 400 V/s) to the carbon fiber electrode. Dopamine release was evoked by optical activation mPOA to VTA fibers using a 60-pulse stimulation (5 ms single pulse duration) at 20 Hz. Therefore, optical stimulation of ChR2-expressing VTA fibers was applied for 3 s starting 5 s after the onset of the voltammetric recording. Recorded voltammetric signals showed an oxidation peak at +0.65 V and a reduction peak at −0.2 V (versus Ag/AgCl reference), as well as characteristic cyclic voltammograms, ensuring that the released chemical was dopamine. Carbon fiber electrodes were calibrated in vitro with known concentrations of dopamine (1.0 and 5.0 µM). Calibrations were done in duplicate and the average value for the current at the peak oxidation potential was used to normalize in vivo signals to dopamine concentration. All voltammetry data were collected and analyzed using TarHeel CV software.

Patch-clamp electrophysiology. Mice were anesthetized with pentobarbital (50 mg/kg) before transcardial perfusion with ice-cold sucrose cutting solution containing the following (in mM): 225 sucrose, 119 NaCl, 1.0 NaHPO4, 4.9 MgCl2, 0.1 CaCl2, 26.2 NaHCO3, 1.25 glucose, 305 mOsM. Brains were then rapidly removed, and coronal sections 300 µm thick were taken using a vibratome (Leica, VT 1200). Sections were then incubated in ACSF (32 °C) containing the following (in mM): 119 NaCl, 2.5 KCl, 1.0 NaHPO4, 1.3 MgCl2, 2.5 CaCl2, 26.2 NaHCO3, 15 glucose, 305 mOsM. After 1 h recovery, slices were constantly superfused with ACSF (32 °C) and visualized using differential interference contrast. Intrinsic excitability of mPOA NTs neurons or mPOA NTs GCaMP6s neurons to characterize the intrinsic excitability of mPOA NTs neurons ex vivo. During recordings, cells were held through slow polarization at −70 mV to control for differences in resting membrane potential between neurons. Next we measured rheobase (the minimum amount of current required for an action potential to fire) by applying a series of short depolarizing sweeps (50 ms at +5-pA steps (starting at 0 pA)) until the recorded neuron fired an action potential. Action potential firing was then examined by applying a series of 800-ms depolarizing sweeps from 0 to 160 pA (10 pA steps for excitability recordings, 20–20 pA steps for GCaMP6s recordings). For GCaMP6s experiments, during patch-clamp recordings we simultaneously recorded GCaMP6s fluorescence through a microscope-mounted camera (OptiMos, QImaging) using imaging software (MicroManager, ImageJ). Videos were subsequently motion-corrected if necessary, and data were extracted using ImageJ.

We also performed whole-cell voltage clamp recordings of fluorescently labeled mPOA NTs-GFP neurons or mPOA NTs-GCAMP6s neurons to measure the intrinsic excitability of mPOA NTs neurons ex vivo. During recordings, cells were held through slow polarization at −70 mV to control for differences in resting membrane potential between neurons. Next we measured rheobase (the minimum amount of current required for an action potential to fire) by applying a series of short depolarizing sweeps (50 ms at +5-pA steps (starting at 0 pA)) until the recorded neuron fired an action potential. Action potential firing was then examined by applying a series of 800-ms depolarizing sweeps from 0 to 160 pA (10 pA steps for excitability recordings, 20–20 pA steps for GCaMP6s recordings). For GCaMP6s experiments, during patch-clamp recordings we simultaneously recorded GCaMP6s fluorescence through a microscope-mounted camera (OptiMos, QImaging) using imaging software (MicroManager, ImageJ). Videos were subsequently motion-corrected if necessary, and data were extracted using ImageJ.

Fluorescence in situ hybridization. Tissue was processed using in situ hybridization to detect mRNA for Nts, Vgat (Slc32a1) and Vglut2 (Slc17a6). Mice were briefly anesthetized with 3.5–4.0% isoflurane mixed with pure oxygen (1 L/min), rapidly decapitated and brains rapidly extracted and flash frozen on dry ice. Cryostat coronal sections 18 µm thick were collected under RNase-free conditions, fixed in 4% PFA for 15 min at 4 °C, dehydrated in serial concentrations of ethanol (50–100%) and processed according to the protocol provided in the RNAscope kit (Advanced Cell Diagnostics, cat. no. 320). Sections were hybridized with the following mixed probes: Nts (Mm-Nts, cat. no. 402441), Vgat (Mm-Slc32a1, cat. no. 319191), Vglut2 (Mm-Slc17a6-C2, 319171) and Ersl (432861-C2 Mm-Esr1-C2) for 2 h at 40 °C, following amplification sections were counterstained with DAPI. For Nts and cre, we performed in situ hybridization using the Affymetrix View RNA 2-Plex Tissue Assay Kit with custom probes for Nts (mouse NM024435, cat. no. V81-16908) and cre (vector, cat. no. HG335171) designed by Affymetrix (Santa Clara, CA).

Immunohistochemistry and histology. To evaluate evoked evoked, female mice received 10 min of photostimulation in the home cage (473 µm, 20 Hz at 3 s) and were euthanized 1 h later. Mice were euthanized with pentobarbital (50 mg/kg, i.p.) and transected with perfused with 0.01 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Tissue was post-fixed overnight at 4 °C in PFA and cryoprotected in 30% sucrose in PBS, and 40-µm-thick cryostat coronal sections were collected. To examine Fos induction, immunohistochemistry was carried out using the following antibodies: c-Fos primary raised in goat (Santa Cruz, cat. no. SC-52g, 1:500 overnight at 4 °C) and secondary (Jackson ImmunoResearch, donkey anti-goat 647, cat. no. 705-606-147, 2 h at room temperature). For TH staining, TH-positive primary in sheep was used (Pel-Freeze, cat. no. PG0101-150, 1:500 overnight at 4 °C). Additional details regarding immunoprocessing procedures were previously described. Tissue slices were slide mounted, counterstained with DAPI, and coverslipped for subsequent confocal microscopy. For all other opticogenetic behavioral cohorts and anatomical tracing experiments, mice were euthanized and perfused, and tissue cryosectioned for verification of viral expression and fiber placement. For in situ cohorts examining the effects of estrogen-induced Nts mRNA, the tissue was processed in duplicate and the same pattern was observed upon replication. For verification of viral expression, all subjects were included in analyses unless post hoc examination of tissue did not confirm the expected viral expression (for example, ChR2, eYFP). This was the case for two mice, and these subjects were removed from the analyses because the procedure is not valid without the proper viral expression. This exclusion criterion was established before conducting any of the present studies.

Confocal microscopy. Images were captured with a confocal microscope (Zeiss LSM 780, Germany) and Zen software with a 20× air and 40× or 63× oil objective. Software settings were optimized for each experiment, but generally z-stacks were collected in less than 1-µm increments throughout the z axis and the maximum intensity projection tiled image was used for representative or quantification purposes.

Protein and mRNA quantification. For quantification of mPOA cells expressing Fos protein or Nts mRNA, an experimenter blind to the treatment group manually tagged cells using ImageJ and the ImageJ Cell Counter plug-in. For quantification of mRNA intensity, an experimenter blind to the treatment condition drew ROIs of the same size around each cell within a single focal plane on the DAPI layer. Then intensity of mRNA expression within each ROI was automatically calculated in ImageJ. These values were then used to determine whether intensity crossed a threshold that designated the cell as positive for the gene of interest (for example, ChR2, eYFP).

Experimental blinding. In all experiments, unless otherwise stated data were analyzed in an automated unbiased manner by a computer software program or custom code. For in vivo imaging and physiology data, software or custom-written
Python codes were used to automate data analysis. The investigator was not blind to within group drug treatment, as it was necessary to administer the appropriate drug (for example, hormone or vehicle) with respect to day or subject. The experimenter was also not blind to the odor treatment, as it was necessary to deliver the appropriate odor.

Statistics. The number of biological replicates in each experiment was at least 3 mice per group for anatomy, 4–8 mice per group for in vivo imaging or physiology, and between 5 and 7 mice per group for behavior. These numbers were chosen on the basis of those used in previous experiments. In addition, for behavioral experiments optogenetic stimulation or inhibition was alternated in repeated time epochs to ensure consistency in behavioral responses. For in vivo imaging experiments, odor was delivered in multiple trials for the same reason and in addition at least 50 neurons were recorded from each mouse; thus for these experiments 4 biological replicates yielded a large sample of data with consistent responses across mice and cells. Data distribution was assumed to be normal, but this was not formally tested. For experiments with hormone or light conditions, two-way ANOVAs were conducted with Bonferroni post hoc analyses. For anatomy experiments and area under the curve for FSCV, unpaired Student’s t-tests were conducted. For cumulative frequency distributions, Kolmogorov-Smirnov tests were conducted. For in vivo odor-evoked imaging a Wilcoxon paired t-test compared cell responses between odors or treatments. For in vivo baseline imaging and optogenetic tests, ANOVAs (RM, one-way, or two-way, as specified in figure legend) were conducted. In all statistical measures a P value <0.05 was considered statistically significant. To obtain behavioral preference indices, calculations were based on prior studies where the amount of time spent investigating the stimulant of interest (for example, opposite-sex odor or social stimulus) and the control side (for example, control odor, saline or same-sex social stimulus) were scored using Noldus and the preference index (PI; ∆t/∑t) for the stimulus was calculated as follows:

\[ PI = \frac{\sum \text{Time spent investigating stimulant} - \sum \text{Time spent investigating control}}{\sum \text{Time spent investigating stimulant} + \sum \text{Time spent investigating control}} \]

A Supplementary Methods Checklist is available.

Data availability. All custom codes have been made publically available at a GitHub repository affiliated with Stuber Laboratory group and this manuscript title (http://www.github.com/stuberlab/). The data that support the findings of this study are available from the corresponding author upon reasonable request.

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In the version of this article initially published, there were errors in data analysis and presentation. The corrected analysis and presentation do not change the results or interpretation of the data. Asterisk definitions have also been added for clarity as noted below. Changes with respect to the number of subjects reflect errors in reporting only and did not affect the data analysis. The percentages in Figure 1b originally reported as 43, 18 and 36% have been changed to 43, 19 and 37%, respectively. In the first paragraph of the Results, “97% of Nt-s labeled cells colocalizing with VTA beads” has been changed to “96% of Nt-s labeled cells colocalizing with VTA beads” and “this subpopulation comprised 33% of all mPOA VTA-projecting neurons” has been changed to “this subpopulation comprised 35% of all mPOA VTA-projecting neurons.” In the legend for Figure 2a, the n value originally reported as 9 has been changed to 9 and 10, and asterisks have been added to read ***P = 0.0006. In Figure 3a, points were misplotted as a result of an error in data analysis. The graph has been replaced. The values originally reported in the legend as t2 = 2.82, P = 0.0368 have been changed to t2 = 5.85, **P = 0.0021. In the Figure 3d legend, the value originally reported as n = 52 cells has been changed to 51. In Figure 3e, the percentage in the male E2 pie chart for excited neurons has been changed from 24 to 23%. Figure 5b originally contained duplicate example traces of calcium transients that were supposed to be taken from three individual neurons; new traces have been supplied. In Figure 5c,d, the asterisks have been changed from *** to **** and defined in the legend as ***P < 0.0001, Bonferroni post hoc test, E2 versus pre and E2 versus post. In the Figure 5d legend, asterisks originally reported as F2,252 = 13.13 has been changed to 17.32. In the Figure 5f legend, asterisks have been added as **P < 0.001, Bonferroni post hoc test. In Figure 5g, the horizontal axis was truncated at 60, resulting in missing data points; the graph has been replaced. In the Figure 5h legend, asterisks have been defined as *P < 0.05, **P < 0.01, ***P < 0.001, Bonferroni post hoc test. In the Figure 6e legend, the degrees of freedom originally reported as F3,36 have been changed to F3,37. In the Figure 6g legend, the value originally reported as eYFP = 6 mice has been changed to eYFP = 7 mice. In the Figure 6h legend, the values originally reported as F3,30 = 9.44, P = 0.0022 have been changed to F3,34 = 15.2, P < 0.0001. In Figure 6i,j, points were misplotted as a result of an error in data analysis, and error bars plotted as s.d. were misidentified in the legend as s.e.m. The data have been replotted, with error bars representing s.e.m. In the Figure 6i legend, the values originally reported as F1,8 = 23.2 have been changed to F1,8 = 63.1. Asterisks have been defined at the end of the Figure 6 legend as *P < 0.05, **P < 0.01, ***P < 0.001, Bonferroni post hoc test. The statistically significant differences in Figure 7b originally indicated by ** have been changed to ***; these have been defined in the legend as ***P < 0.001, Bonferroni post hoc test. In Figure 7c, a data point in the E2 group was missing; the graph has been replaced. An asterisk has also been added in the Figure 7e legend to read *P = 0.016. In Figure 8c, data were misplotted as a result of errors in analysis. The graph has been replaced, and the statistically significant differences originally indicated by * have been changed to **. The values originally reported in the legend as F4,42 = 4.20, P = 0.0112 have been changed to F4,21 = 6.82, P = 0.0011. In Figure 8d, data were misplotted as a result of an error in analysis. The graph has been replaced, and the values originally reported in the legend as F4,42 = 8.33, P = 0.0003 have been changed to F4,21 = 6.35, P = 0.0016. In Figure 8e, data were misplotted as a result of an error in analysis. The graph has been replaced, and the values originally reported in the legend as F4,42 = 60.0, P = 0.6622 have been changed to F4,21 = 1.33, P = 0.29. In Figure 8g, the vertical axis was truncated at 250, resulting in missing data points; the graph has been replaced, and the statistically significant differences originally indicated by * have been changed to **. The values originally reported in the legend as F1,12 = 7.92 as a result of an error in manuscript preparation have been changed to F1,12 = 7.15. In Figure 8h, the vertical axis was truncated at –0.4 and 0.8, resulting in missing data points, and data were misplotted as a result of an error in analysis. The graph has been replaced, and the statistically significant differences originally indicated by ** have been changed to ***. The values originally reported in the legend as F1,12 = 7.15, P = 0.0200 have been changed to F1,12 = 9.8, P = 0.009. In Figure 8j, the vertical axis was truncated at 200, resulting in missing data points. The graph has been replaced, and the test statistic originally described in the legend as F3,33 has been changed to F3,34. In Figure 8k, the vertical axis was truncated at –0.4 and 0.4, resulting in missing data points, and data were misplotted as a result of an error in analysis. The graph has been replaced, and the statistically significant differences originally indicated by *** have been changed to **. The value originally reported in the legend as n = 7 has been changed to control n = 6 and NpHR n = 7.

In Supplementary Figure 5b–d,f,g, points were misplotted as a result of errors in data analysis and figure preparation; the graphs have been replaced. The values originally reported in the legend to Supplementary Figure 5b as t1,11 = 1.71 p = 0.1042, n = 8 mice have been changed to t1,9 = 1.09 p = 0.3061, n = 10 mice. The values originally reported in the legend to Supplementary Figure 5c–e as n = 8 have been changed to n = 7-10. The values originally reported in the legend to Supplementary Figure 5f as F4,37 = 3.61, p = 0.0137, n = 8 mice have been changed to F4,37 = 3.34, p = 0.0196, n = 7-10 mice. The values originally reported in the legend to Supplementary Figure 5g as F4,37 = 1.74, p = 0.1621, n = 8 mice have been changed to F4,37 = 2.01, p = 0.1131, n = 7-10 mice. In Supplementary Figure 6c–e, points were misplotted as a result of an error in data analysis; the graphs have been replaced. The values originally reported in the legend to Supplementary Figure 6c as F2,15 = 17.03, p = 0.0002 have been changed to F2,17 = 35.61, p = 0.0003, and the statistically significant differences originally indicated by *** for Veh have been changed to ** for E2 and *** for E2-P4, with asterisks defined in the legend as Veh. vs. E2. **P = 0.004; Veh. vs. P4, ***P = 0.0003. The values originally reported in the legend to Supplementary Figure 6d as F2,15 = 11.32, p = 0.0010 have been changed to F2,17 = 27.86, p = 0.0008, and the statistically significant differences originally indicated by * for E2 have been changed to **, with asterisks defined in the legend as Veh. vs. E2. **P = 0.009; Veh. vs. P4, ***P = 0.00019. The values originally reported in the legend to Supplementary Figure 6e as F2,15 = 00.48, p = 0.6268 have been changed to F2,17 = 1.76, p = 0.238. The values originally reported in the legend to Supplementary Figure 10b–f as n = 6 have been changed to n = 5-6. The values originally reported in the legend to Supplementary Figure 10c as F4,26 = 1.04 p = 0.9322 have been changed to F4,26 = 0.21 p = 0.9322. The degrees of freedom originally reported in the legend to Supplementary Figure 10f as F2,15 = 11.04 have been changed to F2,13. The test description
and values originally reported in the legend to Supplementary Figure 10i as One-Way ANOVA, interaction $F_{2,13} = 0.72$ $p = 0.5018$ have been changed to One-Way ANOVA, $F_{2,17} = 0.15$ $p = 0.8640$. The test name and values originally reported in the legend to Supplementary Figure 10j as One-Way ANOVA, interaction $F_{2,22} = 9.09$ $p = 0.9099$, $n = 7$ have been changed to Two-Way ANOVA, interaction $F_{2,22} = 0.47$ $p = 0.9543$, $n = 6-7$. The values originally reported in the legend to Supplementary Figure 10k,l as One-Way ANOVA, interaction $F_{2,22} = 1.29$ $p = 0.2953$, $n = 7$ have been changed to Two-Way ANOVA, interaction $F_{2,22} = 0.69$ $p = 0.5142$, $n = 6-7$. The values originally reported in the legend to Supplementary Figure 10m as One-Way ANOVA, interaction $F_{2,22} = 0.686$ $p = 0.5142$, $n = 7$ have been changed to Two-Way ANOVA, interaction $F_{2,22} = 0.405$ $p = 0.6719$, $n = 6-7$. The test name and values originally reported in the legend to Supplementary Figure 10n as Paired t-test, $t_{1,10} = 0.34$ $p = 0.7395$, $n = 8$ have been changed to un-paired t-test, $t_{1,10} = 0.70$ $p = 0.4983$, $n = 6$. The errors have been corrected in the HTML and PDF versions of the article.
Erratum: Hormonal gain control of a medial preoptic area social reward circuit

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In the version of this article initially published, images were pixelated as a result of the production processes used to create the online version. The images have been replaced in the HTML and PDF versions of the article.