Report

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Enhanced Male-Evoked Responses in the Ventromedial Hypothalamus of Sexually Receptive Female Mice

Highlights

- We performed single-unit recordings in female mice interacting with conspecifics
- Social stimuli activate female VMHvl neurons, with preference to male stimuli
- The activity of most VMHvl neurons is modulated throughout social interactions
- Male-evoked VMHvl responses are enhanced during the sexually receptive state

Authors

Kensaku Nomoto, Susana Q. Lima

Correspondence

susana.lima@neuro.fchampalimaud.org

In Brief

Nomoto and Lima show that neural responses to male conspecifics in the ventromedial hypothalamus are enhanced in sexually receptive female mice during initial social interactions. This enhancement could drive gender-specific and reproductive state-dependent sociosexual behavior.

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Report

Enhanced Male-Evoked Responses in the Ventromedial Hypothalamus of Sexually Receptive Female Mice

Kensaku Nomoto[1] and Susana Q. Lima[1,*]
[1]Champalimaud Neuroscience Programme, Champalimaud Centre for the Unknown, Av. de Brasilia, 1400-038 Lisbon, Portugal

Summary

Social encounters often start with routine investigatory behaviors before developing into distinct outcomes, such as affiliative or aggressive actions. For example, a female mouse will initially engage in investigatory behavior with a male but will then show copulation or rejection, depending on her reproductive state. To promote adaptive social behavior, her brain must combine internal ovarian signals and external social stimuli, but little is known about how socially evoked neural activity is modulated across the reproductive cycle [1]. To investigate this, we performed single-unit recordings in the ventrolateral region of the ventromedial hypothalamus (VMHvl) in freely behaving, naturally cycling, female mice interacting with conspecifics of both genders. The VMHvl has been implicated in rodent sociosexual behavior [2, 3]: it has access to social sensory stimuli [4–8] and is involved in aggression and mating [9–11]. Furthermore, many VMHvl neurons express ovarian hormone receptors [12, 13], which play a central role in female sociosexual behavior [14–16]. We found that a large fraction of VMHvl neurons was activated in the presence of conspecifics with preference to male stimuli and that the activity of most VMHvl neurons was modulated throughout social interactions rather than in response to specific social events. Furthermore, neuronal responses to male, but not female, conspecifics in the VMHvl were enhanced during the sexually receptive state. Thus, male-evoked VMHvl responses are modulated by the reproductive state, and VMHvl neural activity could drive gender-specific and reproductive state-dependent sociosexual behavior.

Results

To study how socially evoked responses of the ventrolateral region of the ventromedial hypothalamus (VMHvl) neurons are modulated by the hormonal reproductive state, we performed single-unit recordings in freely behaving female mice while they interacted with males or females (Figure 1A). Since subject animals had regular estrous cycles, we were able to investigate neuronal responses during two different reproductive states: estrous (sexually receptive) and non-estrous (not receptive). Because we were interested in the investigatory phase of social behavior, copulation was not allowed during the chronic single-unit recording experiments in order to avoid pregnancy or pseudopregnancy (which would lead to profound neuroendocrinological changes and cause the female to be in a different physiological state [17]).

*Correspondence: susana.lima@neuro.fchampalimaud.org

Females Interact Differently with Males and Females, but There Is No Effect of the Reproductive State on Initial Investigatory Behavior

We first investigated the subject female's behavior during social interactions. We examined several behavioral measures, including the mean duration of the interaction (Figures 1B and S1; see Experimental Procedures for a list of behavioral measures) and behavioral occupancy maps that indicate the position of the nose of the subject animal relative to the stimulus (Figures 1C–1E; it indicates how the subject animal is interacting with the stimulus animal). The subject female showed different behavioral patterns depending on the gender of the stimulus animal. The subject female spent more time interacting with males than with females (Figure 1B) and performed less anogenital investigation toward males than toward females (Figures 1D and 1E). In contrast, there was no effect of the reproductive state of the subject female on her behavior during social interactions (Figures 1B, 1D, 1E, and S1). The behavior of the stimulus animal was also not modulated by the reproductive state of the subject female, except for a tendency of stimulus males to approach the female with shorter latencies when she was sexually receptive (Figure S1). Taken together, we conclude that the reproductive state of the subject female does not generally affect initial investigatory behavior.

Social Behaviors before Copulation Do Not Predict whether Females Are Sexually Receptive

To investigate how social interactions between females and males would unroll depending on the reproductive state, we conducted separate behavioral experiments during which copulation was allowed (~20–60 min). We confirmed the finding that initial investigatory behavior was not modulated by the reproductive state (Figures 2A and S2A). Furthermore, we found that social behaviors involving the estrous females were not significantly different from those involving the non-estrous females during the entire interaction (Figures 2A and S2B). This is in sharp contrast with the observation that the reproductive state heavily influenced female sexual behavior (Figure 2B). Whereas none of six non-estrous females copulated with males, five out of six estrous females showed copulatory behavior (mean latency to the first intromission, 1,055.8 ± 198.9 s, n = 5; three of these females received ejaculations). Although the males tended to attempt more mountings toward the estrous females (Figure 2C), they did so only after 30 min from the start of the interaction (Figure 2A). These results suggest that social behaviors before copulation do not predict whether females will later engage in copulation.

VMHvl Neurons Have Variable Gender Selectivity and Modest Spatial Information around the Interaction Partner

We recorded 89 individual VMHvl units (16 neurons were recorded in the two different reproductive states, constituting a total of 105 recordings; Figures 3A and S3). The baseline firing rate of VMHvl neurons was low (2.65 ± 0.30 Hz; n = 89), and a large fraction of neurons responded to the stimulus animal with preference to male stimuli (72% and 46% of 105

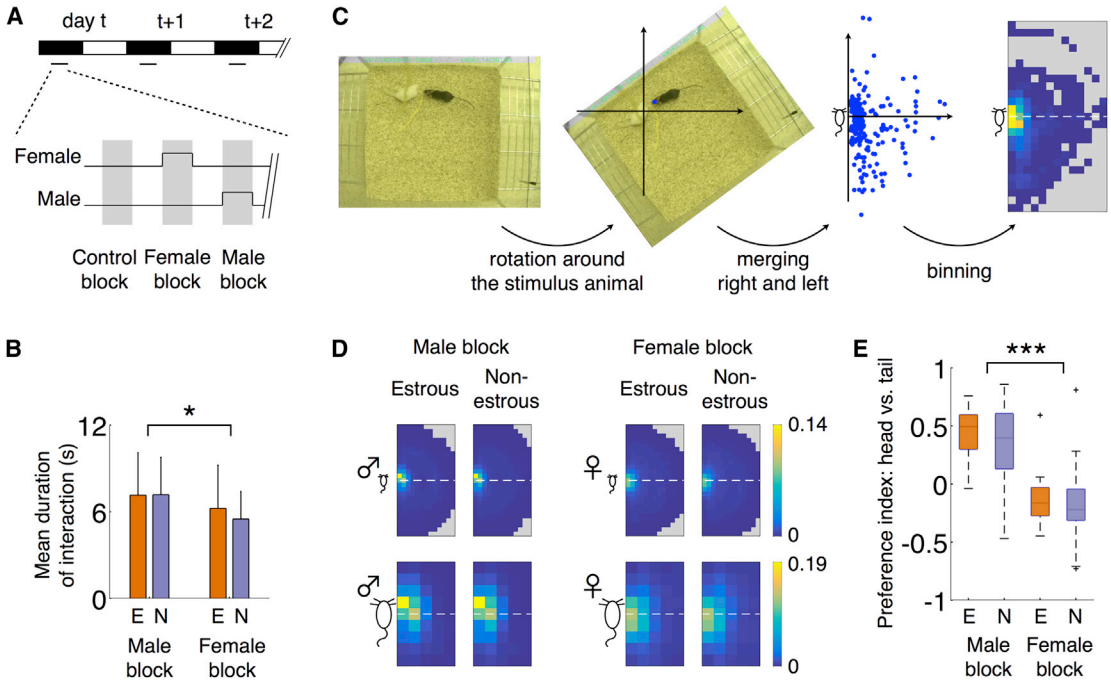

Figure 1. Female Mice Exhibit Gender-Specific Behavior with No Influence of the Reproductive State

Orange indicates estrous (n = 14 sessions); blue indicates non-estrous (n = 41 sessions). Data represent mean ± SEM.

(A) Schema of the behavioral paradigm.

(B) An example of behavioral measures: mean duration of interaction. *p < 0.05 for main effect of the gender of the stimulus animal, two-way ANOVA.

(C) Schematic representations of how to create behavioral occupancy maps.

(D) Behavioral occupancy maps around the stimulus animal. Color indicates the probability of the subject female being at that particular location. The maps in the lower row are zoomed-in images of the maps in the upper row. The cartoon of the reference mouse is shown next to the map (its size is scaled to the map size).

(E) Head-tail preference index (1 represents preference toward the head area, −1 represents preference toward the tail area, and 0 represents no preference). ***p < 0.001 for main effect of the gender of the stimulus animal, two-way ANOVA.

See also Figure S1.

recordings for males and females, respectively; p = 0.006, χ^2 test). To understand how neuronal activity is modulated during social interactions, we constructed firing rate maps in which neural firing rates were mapped onto the behavioral occupancy maps. Figures 3B–3D show example response patterns of three VMHvl neurons that were activated by males, females, and both genders, respectively. To quantify gender selectivity, we performed receiver operating characteristic (ROC) analysis [18, 19]. We used the area under the ROC curve (auROC; A versus B) as an index for gender selectivity with a range from 0 to 1, where 0 represents "preference to B," 1 represents "preference to A," and 0.5 represents no selectivity. At the population level, VMHvl neurons had variable responses to both males and females (Figure 3E; average auROC; male: 0.58 ± 0.02; female: 0.56 ± 0.01). Across neurons, we found that male selectivity was strongly correlated with female selectivity (Spearman's r = 0.66, p < 0.001, Spearman's rank correlation test). We did not find evidence of anatomical segregation: the average auROC did not change along the anteroposterior axis (p > 0.10, one-way ANOVA; Figures S3E and S3F).

The activity of VMHvl neurons also differed in spatial selectivity around the stimulus animal, ranging from diffuse firing (e.g., Figure 3B) to localized firing near the stimulus animal (e.g., Figure 3C; Movie S1; activated when sniffing the anogenital area of the stimulus female). To assess spatial selectivity around the interaction partner, the information rate was computed for firing rate maps [20]. Since firing rate maps are constructed around the interaction partner, high spatial information rate could result from a situation where the neuron is activated when the subject female performs a specific behavior on the stimulus animal. The results show that VMHvl neurons carry modest spatial information (Figure 3F; average, 0.52 bit/s). Thus, while a minority of VMHvl neurons were activated during specific social interactions, the activity of most VMHvl neurons was modulated independently of the relative position to the stimulus animal. Additionally, we found that the neurons recorded at the most anterior level had significantly lower information rate compared to those recorded at more posterior levels (p < 0.05, post hoc Tukey-Kramer test after significant one-way ANOVA; Figure S3G).

Male-Evoked VMHvl Responses Are Enhanced during Sexually Receptive State

We investigated the effect of the reproductive state on the activity of VMHvl neurons by comparing the activity of neurons recorded in the estrous state to those recorded in the non-estrous state. Figure 4A shows the response of a neuron recorded in both states that was modulated by the reproductive state. This neuron showed male-evoked, but not female-evoked, firing specifically in the estrous state. At the population level, VMHvl neurons showed significantly greater male-evoked firing during the estrous state than during

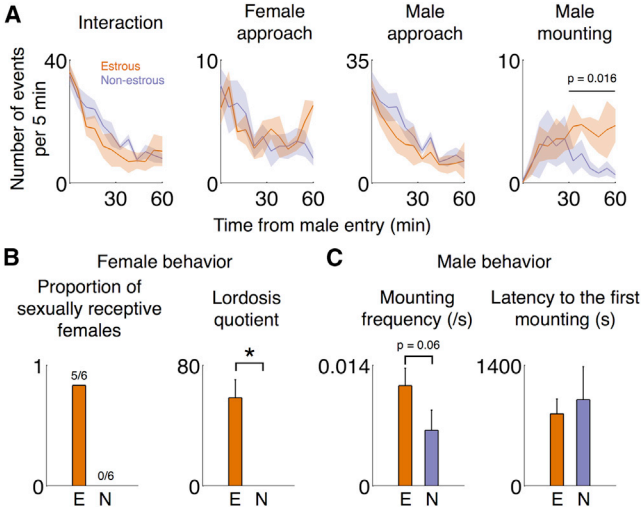

Figure 2. Social Behaviors before Copulation Are Not Correlated with Female Sexual Receptivity

Orange indicates estrous (n = 6 animals); blue indicates non-estrous (n = 6 animals). Data represent mean ± SEM.

(A) Number of events per 5 min as a function of time. Males showed more mounting behavior toward estrous females after 30 min (p = 0.016, Mann-Whitney U test).

(B) Female sexual behavior: the proportion of female mice that copulated with males and lordosis quotient (the ratio of the number of intromission to the number of mounting attempts multiplied by 100). *p < 0.05, t test.

(C) Male mounting behavior: frequency and latency to the first mounting.

See also Figure S2.

non-estrous states (Figure 4B; 4.90 ± 0.90 Hz versus 2.90 ± 0.43 Hz; p = 0.026, permutation test). The proportion of male-responsive neurons was also significantly higher during the estrous state compared to during the non-estrous state (Figure 4C; p = 0.012, χ^2 test). In contrast, we observed no modulation by the reproductive state of neuronal responses to the stimulus female (Figures 4B and 4C). Baseline firing rates in the absence of stimulus animals were slightly higher in the estrous state, but not statistically greater than those in the non-estrous states (3.41 ± 0.57 Hz versus 2.38 ± 0.32 Hz, p = 0.095, permutation test). Finally, reexamination of gender and spatial selectivity showed no effect of the reproductive state on these measures (Figure S4).

Discussion

Our results show that the sexually receptive state in females is correlated with an enhancement in the neuronal response of VMHvl neurons to males during the investigatory phase of social behavior. Although some pioneering work investigated copulation-related activity of hypothalamic neurons in behaving females during the sexually receptive state [21–23], little is known about how ovarian hormones influence neuronal activity during social interactions [1]. To our knowledge, the present finding is the first electrophysiological evidence that the activity of hypothalamic neurons is modulated during social encounters, in a gender-specific and reproductive state-dependent manner. Since initial investigatory behavior is essential to many, if not all, social behaviors, our findings will have implications not only for female sexual behavior but also for other social behaviors, such as aggression, social fear, and male copulatory behavior, where the contribution of VMHvl has recently been reported [24–27].

We found that female behavior was differently modulated by the gender of the stimulus animal during the initial investigatory phase, indicating that the animals could discriminate the gender of their partners and change their behavior accordingly. Surprisingly, we did not find significant effects of the reproductive state on the initial investigatory behavior of both subject and stimulus animals: females that had copulation with males showed similar social behavior compared to the females that rejected males' mounting attempts. We cannot rule out the existence of other channels of communication, including vocalizations [28, 29], or the execution of more subtle behaviors that were not detected by our behavioral analysis. However, our finding is consistent with previous work showing that female attraction behavior toward males is not modulated by hormonal manipulations [30].

We found gender-specific responses in VMHvl neurons. Particularly, there were more male-responsive neurons in the female VMHvl than female-responsive neurons. This is consistent with previous findings showing that VMHvl neurons are activated with preference to the opposite gender in both males and females [24, 31, 32], which may reflect biological significance. We also found that most VMHvl neurons have modest spatial information around the interaction partner. This indicates that activity of VMHvl neurons is modulated throughout social interactions rather than in response to specific social events, which is similar to previous findings in males, where VMHvl neurons encode aggressive motivation in a persistent manner [24, 27]. The analysis of the recording sites within the VMHvl suggests that there might be anatomical segregation of spatial selectivity, but not gender selectivity. This might reflect a difference in connectivity along the anteroposterior axis [33, 34]. Taken together, our results support the idea that VMHvl activity encodes the motivational state of the animal [24, 27].

Our main finding is that male-evoked VMHvl responses are enhanced during the sexually receptive state. This result suggests the existence of gender-specific inputs to VMHvl neurons and the ability of these inputs to be differentially modulated by ovarian hormones. The gender-specific inputs might be transmitted to the VMHvl through the main olfactory bulb-to-medial amygdala pathway [32]. In addition, the VMHvl has rich expression of ovarian hormone receptors [12, 13], granting this hypothalamic nucleus the capability of being modulated by ovarian hormones through multiple mechanisms: increased neuronal responsiveness (as shown in vitro [35] and in anesthetized animals [36]) and changes in dendritic morphology [37–39]. Changes in dendritic morphology, in particular, the expansion of the dendritic harbor of VMHvl neurons, may allow different neuromodulatory inputs (e.g., oxytocin) to alter the activity of specific cell populations in the VMHvl [39]. Accordingly, gender-specific and reproductive state-dependent enhancement of VMHvl activity could be achieved by ovarian hormone-induced facilitation of afferent pathways that are activated preferentially by the opposite gender. An important future step will be to use optogenetic tools [40–42] to determine which neuronal populations within the VMHvl change their properties to male stimuli across the reproductive cycle and how those neurons impact downstream areas such as the periaqueductal gray. Neurons expressing ovarian

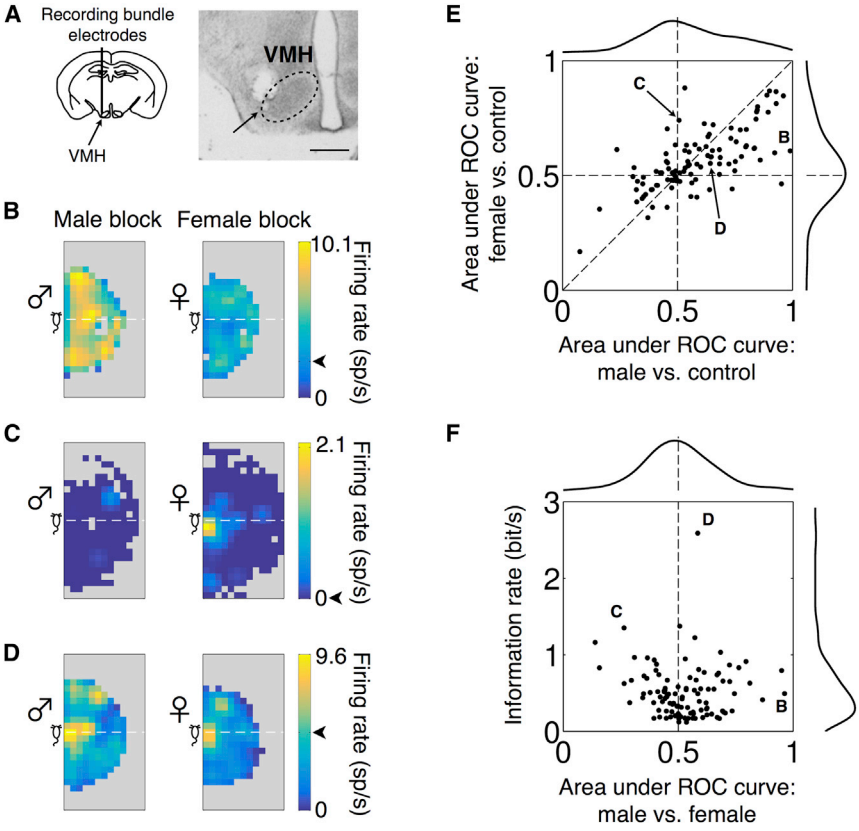

Figure 3. Electrophysiological Properties of VMHvl Neurons during Social Investigation

(A) A schematic drawing illustrating the recordings and a Nissl-stained section showing a representative recording site within the VMHvl. The dashed line delineates the VMH. The arrow indicates the microlesion. The scale bar represents 500 μm.

(B–D) Firing maps of three example neurons with different response patterns. Color indicates the mean firing rate at the particular location. The baseline firing rate is indicated by an arrowhead in the color bar. A neuron in (B) was activated diffusely during the male block; a neuron in (C) showed localized responses at the anogenital area of the stimulus female (see also Movie S1); and a neuron in (D) had localized responses at the anogenital area of the stimulus animal of both genders.

(E) Gender selectivity: male selectivity is plotted against female selectivity. Estimated densities are shown on the top and the right. Example neurons are indicated by the letters.

(F) Spatial selectivity around the interaction partner: the spatial information rate is plotted against male-female selectivity. Estimated densities are shown on the top and the right. Example neurons are indicated by the letters.

See also Figure S3.

hormone receptors will provide a particularly interesting target [16, 26].

Experimental Procedures

For details, please see the Supplemental Experimental Procedures. All procedures were reviewed and performed in accordance with the Champalimaud Centre for the Unknown Ethics Committee guidelines and approved by the Portuguese Veterinary General Board. Single-unit recordings were performed in the VMHvl of seven female C57BL/6 mice during the dark period of light/dark cycle (Omniplex system, Plexon; a movable 16-channel electrode drive, Innovative Neurophysiology). The estrous phase was determined by daily vaginal lavage. BALB/c or FVB/N mice were used as stimulus animals. Our experimental paradigm consisted of several blocks (0.5–5 min) during which the subject animal was allowed to interact with a male stimulus, a female stimulus, or an object. No stimulus was presented during the control block. We did not allow the animals to copulate during the recording experiment. The stimulus animal was removed if it showed excessive aggression or started to copulate with the subject female. The positions of two animals were detected by custom-made software. Three behaviors were annotated (approach, withdrawal, interaction) [43]. Four measures were computed for each behavior (frequency, duration, latency, and the percentage of time engaged). To analyze the behavior of the subject female during social interactions, occupancy maps were created around the interaction partner (Figure 1C). Head-tail preference index was defined as $(h - t)/(h + t)$, where h and t indicate the probability of the subject female being in the two spatial bins around the head and tail of the stimulus animal, respectively. The baseline firing rate was defined as the mean firing rate during the control block. A single neuron was considered responsive if the distribution of the interspike intervals during the stimulus block was significantly different from that during the control block ($p < 0.01$, two-tailed Kolmogorov-Smirnov test). Responsive neurons were further classified into two types based on mean firing rate: excited and inhibited types. To quantify gender selectivity, we performed ROC analysis [18, 19]. To quantify spatial selectivity, we computed information rate about the firing rate map [20]. To examine the effect of the reproductive state on the firing rate, we performed a permutation test on the difference in the mean firing rate between the two reproductive states. In order to examine how social interactions between females and males develop depending on the reproductive state, we conducted separate behavioral experiments, where copulation was allowed (~20–60 min). Six behaviors were annotated manually (interaction, female approach, male approach, male mounting, male intromission, male ejaculation). Lordosis quotient was defined as the ratio of the number of intromissions to the number of mounting attempts multiplied by 100. Data were represented as mean ± SEM in the main text.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.12.048.

Author Contributions

K.N. and S.Q.L. designed the experiments and wrote the manuscript. K.N. performed and analyzed the experiments.

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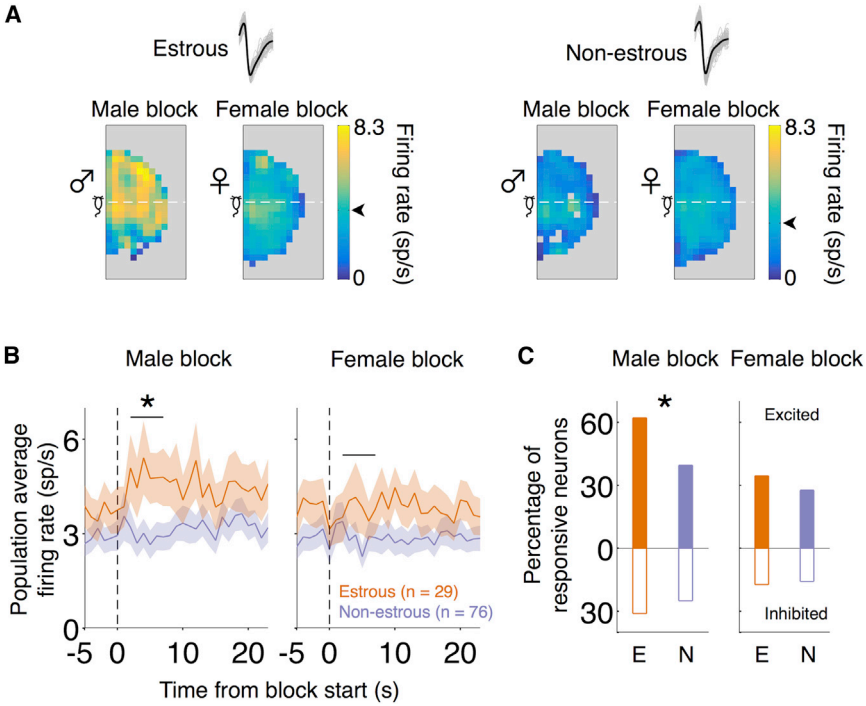

Figure 4. VMHvl Activity in Response to Males Is Modulated by the Reproductive State

Orange indicates estrous (n = 29 recordings); blue indicates non-estrous (n = 76 recordings).

(A) Firing maps of two successive recordings from an example neuron showing response modulation by the reproductive state. The legends are the same as in Figure 3B. For each recording, the average waveform (black) and 50 evenly sampled waveforms (gray) are shown (point-by-point Pearson's r = 0.98 between two average waveforms).

(B) Population activity of VMHvl neurons aligned with the block start. Lines with shades show mean ± SEM. Stimulus-evoked responses were defined as the mean firing rate during the interval from 2 s to 7 s after the block start indicated by a black bar above population activity (male: 4.90 Hz and 2.90 Hz, p = 0.026; female: 3.79 Hz and 2.86 Hz, p = 0.25; stimulus-evoked responses of the estrous and non-estrous states, and p value from permutation test). *p < 0.05.

(C) Proportion of stimulus-responsive neurons. Stimulus responsiveness was evaluated by comparing distributions of interspike intervals (p < 0.01, Kolmogorov-Smirnov test). Filled bars indicate the proportion of stimulus-excited type, and open bars indicate the proportion of stimulus-inhibited type. *p < 0.05; χ^2 test.

See also Figure S4.

References

1. Bobrov, E., Wolfe, J., Rao, R.P., and Brecht, M. (2014). The representation of social facial touch in rat barrel cortex. Curr. Biol. *24*, 109–115.
2. Pfaff, D.W. (1980). Estrogens and Brain Function: Neural Analysis of a Hormone-Controlled Mammalian Reproductive Behavior (New York: Springer Verlag).
3. Blaustein, J.D., and Erskine, M.S. (2002). Feminine sexual behavior: cellular interation of hormonal and afferent information in the rodent forebrain. In Hormones, Brain and Behavior, Volume One, D.W. Pfaff, A.P. Arnold, S.E. Fahrbach, A.M. Etgen, and R.T. Rubin, eds. (San Diego: Academic Press), pp. 139–214.
4. Pfaus, J.G., Kleopoulos, S.P., Mobbs, C.V., Gibbs, R.B., and Pfaff, D.W. (1993). Sexual stimulation activates c-fos within estrogen-concentrating regions of the female rat forebrain. Brain Res. *624*, 253–267.
5. Tetel, M.J., Getzinger, M.J., and Blaustein, J.D. (1994). Estradiol and progesterone influence the response of ventromedial hypothalamic neurons to tactile stimuli associated with female reproduction. Brain Res. *646*, 267–272.
6. Pfaus, J.G., Marcangione, C., Smith, W.J., Manitt, C., and Abillamaa, H. (1996). Differential induction of Fos in the female rat brain following different amounts of vaginocervical stimulation: modulation by steroid hormones. Brain Res. *741*, 314–330.
7. Halem, H.A., Cherry, J.A., and Baum, M.J. (1999). Vomeronasal neuroepithelium and forebrain Fos responses to male pheromones in male and female mice. J. Neurobiol. *39*, 249–263.
8. Georgescu, M., Sabongui, C., Del Corpo, A., Marsan, L., and Pfaus, J.G. (2009). Vaginocervical stimulation induces Fos in glutamate neurons in the ventromedial hypothalamus: attenuation by estrogen and progesterone. Horm. Behav. *56*, 450–456.
9. Mathews, D., and Edwards, D.A. (1977). The ventromedial nucleus of the hypothalamus and the hormonal arousal of sexual behaviors in the female rat. Horm. Behav. *8*, 40–51.
10. Pfaff, D.W., and Sakuma, Y. (1979). Deficit in the lordosis reflex of female rats caused by lesions in the ventromedial nucleus of the hypothalamus. J. Physiol. *288*, 203–210.
11. Kruk, M.R., Van der Poel, A.M., Meelis, W., Hermans, J., Mostert, P.G., Mos, J., and Lohman, A.H. (1983). Discriminant analysis of the localization of aggression-inducing electrode placements in the hypothalamus of male rats. Brain Res. *260*, 61–79.
12. Simerly, R.B., Chang, C., Muramatsu, M., and Swanson, L.W. (1990). Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J. Comp. Neurol. *294*, 76–95.
13. Shughrue, P.J., Lane, M.V., and Merchenthaler, I. (1997). Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system. J. Comp. Neurol. *388*, 507–525.
14. Musatov, S., Chen, W., Pfaff, D.W., Kaplitt, M.G., and Ogawa, S. (2006). RNAi-mediated silencing of estrogen receptor α in the ventromedial nucleus of hypothalamus abolishes female sexual behaviors. Proc. Natl. Acad. Sci. USA *103*, 10456–10460.
15. Spiteri, T., Musatov, S., Ogawa, S., Ribeiro, A., Pfaff, D.W., and Ågmo, A. (2010). Estrogen-induced sexual incentive motivation, proceptivity and receptivity depend on a functional estrogen receptor α in the ventromedial nucleus of the hypothalamus but not in the amygdala. Neuroendocrinology *91*, 142–154.
16. Yang, C.F., Chiang, M.C., Gray, D.C., Prabhakaran, M., Alvarado, M., Juntti, S.A., Unger, E.K., Wells, J.A., and Shah, N.M. (2013). Sexually dimorphic neurons in the ventromedial hypothalamus govern mating in both sexes and aggression in males. Cell *153*, 896–909.
17. Erskine, M.S., Lehmann, M.L., Cameron, N.M., and Polston, E.K. (2004). Co-regulation of female sexual behavior and pregnancy induction: an exploratory synthesis. Behav. Brain Res. *153*, 295–315.
18. Green, D.M., and Swets, J.A. (1966). Signal Detection Theory and Psychophysics (New York: Wiley).
19. Feierstein, C.E., Quirk, M.C., Uchida, N., Sosulski, D.L., and Mainen, Z.F. (2006). Representation of spatial goals in rat orbitofrontal cortex. Neuron *51*, 495–507.
20. Skaggs, W.E., McNaughton, B.L., Gothard, K., and Markus, E.J. (1993). An information-theoretic approach to deciphering the hippocampal code. In Advances in Neural Information Processing Systems 5, S.J. Hanson, J.D. Cowan, and C.L. Giles, eds. (San Mateo: Morgan Kaufmann), pp. 1030–1037.
21. Aou, S., Oomura, Y., and Yoshimatsu, H. (1988). Neuron activity of the ventromedial hypothalamus and the medial preoptic area of the female monkey during sexual behavior. Brain Res. *455*, 65–71.
22. Rose, J.D. (1990). Changes in hypothalamic neuronal function related to hormonal induction of lordosis in behaving hamsters. Physiol. Behav. *47*, 1201–1212.
23. Kato, A., and Sakuma, Y. (2000). Neuronal activity in female rat preoptic area associated with sexually motivated behavior. Brain Res. *862*, 90–102.

24. Lin, D., Boyle, M.P., Dollar, P., Lee, H., Lein, E.S., Perona, P., and Anderson, D.J. (2011). Functional identification of an aggression locus in the mouse hypothalamus. Nature *470*, 221–226.
25. Silva, B.A., Mattucci, C., Krzywkowski, P., Murana, E., Illarionova, A., Grinevich, V., Canteras, N.S., Ragozzino, D., and Gross, C.T. (2013). Independent hypothalamic circuits for social and predator fear. Nat. Neurosci. *16*, 1731–1733.
26. Lee, H., Kim, D.W., Remedios, R., Anthony, T.E., Chang, A., Madisen, L., Zeng, H., and Anderson, D.J. (2014). Scalable control of mounting and attack by Esr1+ neurons in the ventromedial hypothalamus. Nature *509*, 627–632.
27. Falkner, A.L., Dollar, P., Perona, P., Anderson, D.J., and Lin, D. (2014). Decoding ventromedial hypothalamic neural activity during male mouse aggression. J. Neurosci. *34*, 5971–5984.
28. Holy, T.E., and Guo, Z. (2005). Ultrasonic songs of male mice. PLoS Biol. *3*, e386.
29. Asaba, A., Okabe, S., Nagasawa, M., Kato, M., Koshida, N., Osakada, T., Mogi, K., and Kikusui, T. (2014). Developmental social environment imprints female preference for male song in mice. PLoS ONE *9*, e87186.
30. Moncho-Bogani, J., Lanuza, E., Lorente, M.J., and Martinez-Garcia, F. (2004). Attraction to male pheromones and sexual behaviour show different regulatory mechanisms in female mice. Physiol. Behav. *81*, 427–434.
31. Martel, K.L., and Baum, M.J. (2009). A centrifugal pathway to the mouse accessory olfactory bulb from the medial amygdala conveys gender-specific volatile pheromonal signals. Eur. J. Neurosci. *29*, 368–376.
32. Kang, N., Baum, M.J., and Cherry, J.A. (2009). A direct main olfactory bulb projection to the 'vomeronasal' amygdala in female mice selectively responds to volatile pheromones from males. Eur. J. Neurosci. *29*, 624–634.
33. Fahrbach, S.E., Morrell, J.I., and Pfaff, D.W. (1989). Studies of ventromedial hypothalamic afferents in the rat using three methods of HRP application. Exp. Brain Res. *77*, 221–233.
34. Canteras, N.S., Simerly, R.B., and Swanson, L.W. (1994). Organization of projections from the ventromedial nucleus of the hypothalamus: a Phaseolus vulgaris-leucoagglutinin study in the rat. J. Comp. Neurol. *348*, 41–79.
35. Kow, L.M., and Pfaff, D.W. (1985). Estrogen effects on neuronal responsiveness to electrical and neurotransmitter stimulation: an in vitro study on the ventromedial nucleus of the hypothalamus. Brain Res. *347*, 1–10.
36. Bueno, J., and Pfaff, D.W. (1976). Single unit recording in hypothalamus and preoptic area of estrogen-treated and untreated ovariectomized female rats. Brain Res. *101*, 67–78.
37. Calizo, L.H., and Flanagan-Cato, L.M. (2000). Estrogen selectively regulates spine density within the dendritic arbor of rat ventromedial hypothalamic neurons. J. Neurosci. *20*, 1589–1596.
38. Madeira, M.D., Ferreira-Silva, L., and Paula-Barbosa, M.M. (2001). Influence of sex and estrus cycle on the sexual dimorphisms of the hypothalamic ventromedial nucleus: stereological evaluation and Golgi study. J. Comp. Neurol. *432*, 329–345.
39. Griffin, G.D., Ferri-Kolwicz, S.L., Reyes, B.A., Van Bockstaele, E.J., and Flanagan-Cato, L.M. (2010). Ovarian hormone-induced reorganization of oxytocin-labeled dendrites and synapses lateral to the hypothalamic ventromedial nucleus in female rats. J. Comp. Neurol. *518*, 4531–4545.
40. Lima, S.Q., Hromádka, T., Znamenskiy, P., and Zador, A.M. (2009). PINP: a new method of tagging neuronal populations for identification during in vivo electrophysiological recording. PLoS ONE *4*, e6099.
41. Gunaydin, L.A., Grosenick, L., Finkelstein, J.C., Kauvar, I.V., Fenno, L.E., Adhikari, A., Lammel, S., Mirzabekov, J.J., Airan, R.D., Zalocusky, K.A., et al. (2014). Natural neural projection dynamics underlying social behavior. Cell *157*, 1535–1551.
42. Tecuapetla, F., Matias, S., Dugue, G.P., Mainen, Z.F., and Costa, R.M. (2014). Balanced activity in basal ganglia projection pathways is critical for contraversive movements. Nat. Commun. *5*, 4315.
43. Kabra, M., Robie, A.A., Rivera-Alba, M., Branson, S., and Branson, K. (2013). JAABA: interactive machine learning for automatic annotation of animal behavior. Nat. Methods *10*, 64–67.

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Supplemental Information

Enhanced Male-Evoked Responses in the Ventromedial Hypothalamus of Sexually Receptive Female Mice

Kensaku Nomoto and Susana Q. Lima

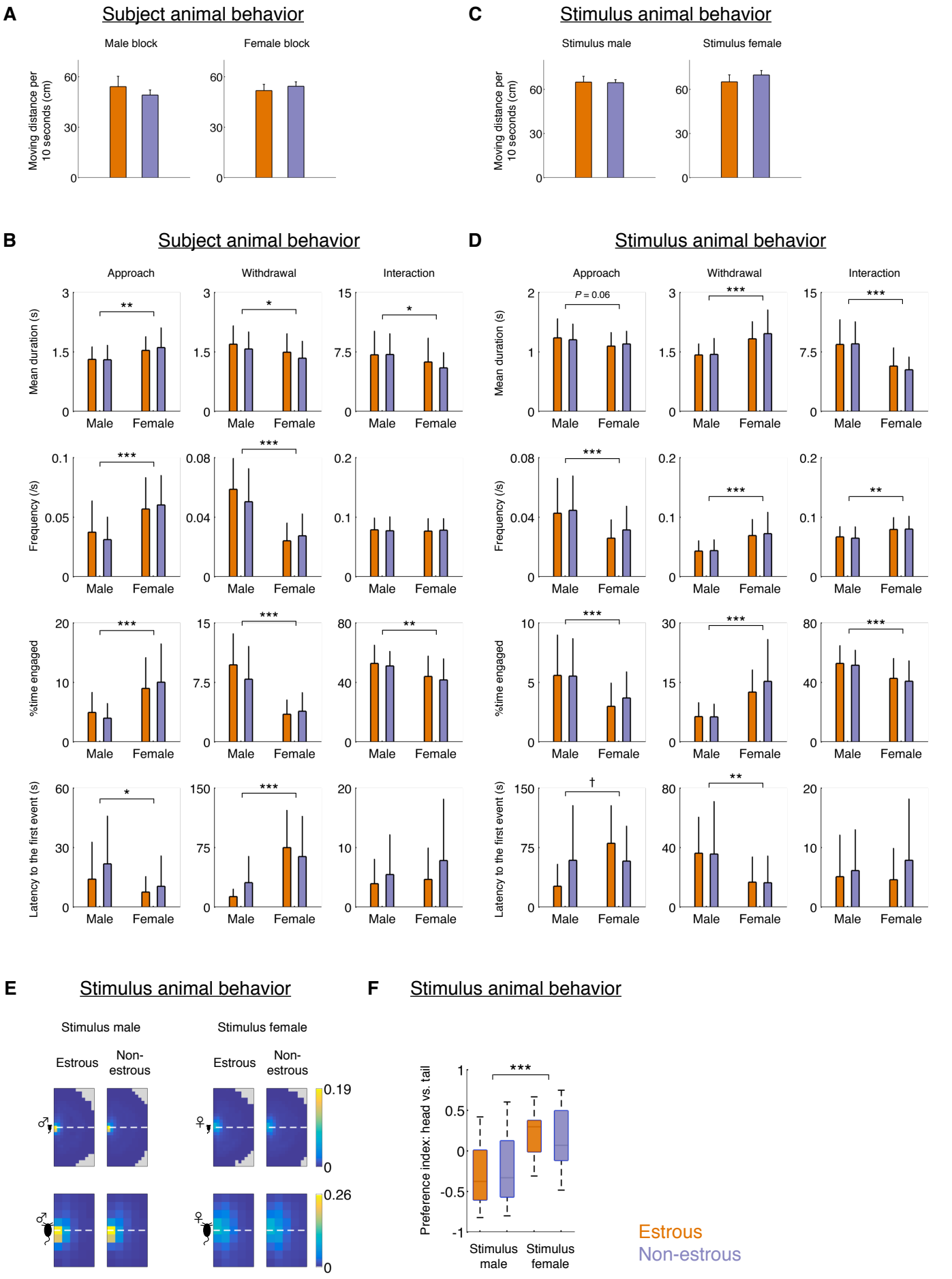
A
Subject animal behavior
Male block
Female block
Moving distance per 10 seconds (cm)
C
Stimulus animal behavior
Stimulus male
Stimulus female
B
Subject animal behavior
D
Stimulus animal behavior
Approach
Withdrawal
Interaction
Mean duration (s)
Frequency (/s)
%time engaged
Latency to the first event (s)
Male
Female
P = 0.06
E
Stimulus animal behavior
Stimulus male
Stimulus female
Estrous
Non-estrous
F
Stimulus animal behavior
Preference index: head vs. tail
Stimulus male
Stimulus female
Estrous
Non-estrous

Figure S1. Effect of the Gender and the Reproductive State on Animals' Behavior, Related to Figure 1. Orange indicates estrous (n = 14 sessions); blue indicates non-estrous (n = 41 sessions). Data represent mean ± SEM.

(A–B) subject animal behavior.

(A) No effect of the reproductive state on the moving distance per 10 seconds during both male and female blocks ($P > 0.10$, t-test).

(B) No effect of the reproductive state on behavioral measures. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for main effect of the gender of the stimulus animal.

(C–F) stimulus animal behavior.

(C) No effect of the reproductive state on the moving distance per 10 seconds during both male and female blocks ($P > 0.10$, t-test).

(D) No effect of the reproductive state on behavioral measures. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for main effect of the gender of the stimulus animal; † $P < 0.05$ for main effect of the gender of the stimulus animal and interaction effect.

(E) Occupancy maps representing the stimulus animal position relative to the subject female. Color indicates the probability for the stimulus animal being at this location. The legends are the same as in Figure 1D.

(F) Effect of the gender of the stimulus animal on head-tail preference index: 1 represents preference towards the head area, −1 represents preference towards the tail area, and 0 represents no preference. *** $P < 0.001$ for main effect of the gender of the stimulus animal.

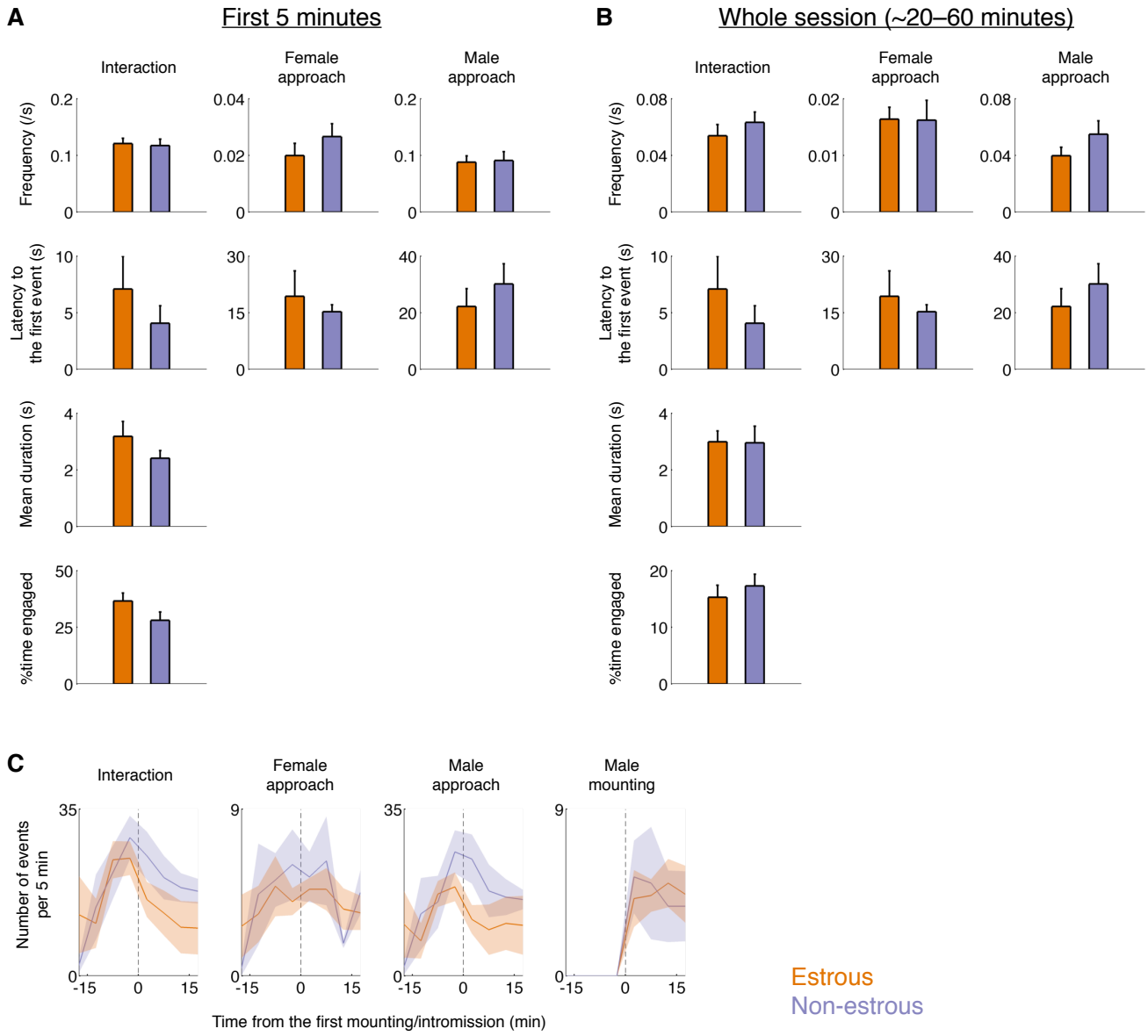

Figure S2. No Effect of the Reproductive State on Animals' Behavior in a Longer Timeframe, Related to Figure 2.

Orange indicates estrous (n = 6 animals); blue indicates non-estrous (n = 6 animals). Data represent mean ± SEM.

(A) No effect of the reproductive state during the first five minutes of the experiments (all comparisons, $P > 0.10$, Mann-Whitney U test).

(B) No effect of the reproductive state during the whole session, which lasted for 20–60 minutes (all comparisons, $P > 0.10$, Mann-Whitney U test).

(C) Number of events per 5 minutes as a function of time from the first mounting (or intromission, if applicable). Note that the data is the same as in Figure 2A.

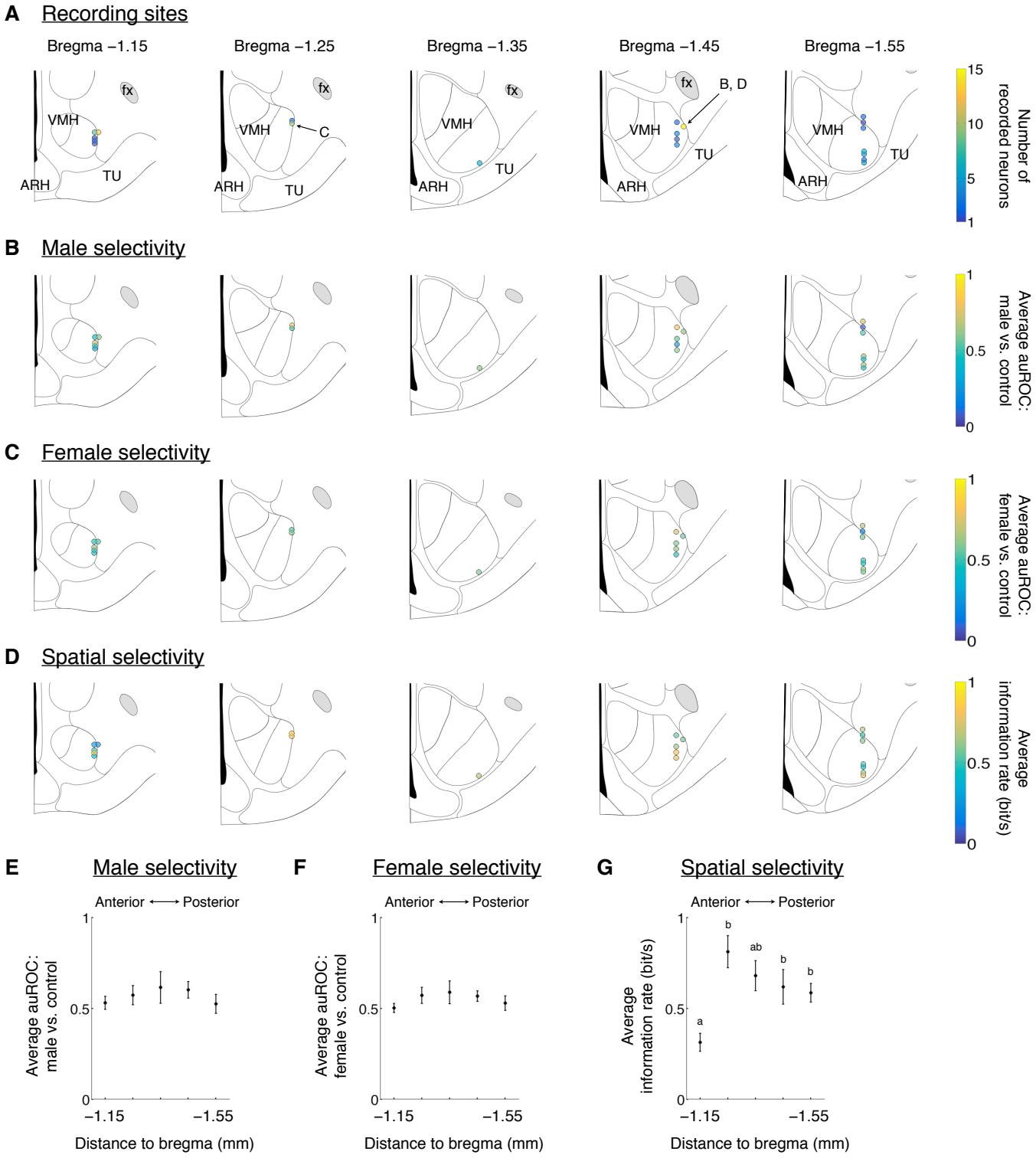

Figure S3. Recording Sites and Neuronal Selectivity, Related to Figure 3.
(A) Histological reconstruction of recording sites. The recording sites of example neurons in Figure 3B, 3C, and 3D are indicated by the letters. Circles are plotted at the center of a single recording site. Coronal brain images are from Allen Brain Atlas. VMH, ventromedial hypothalamus; ARH, arcuate hypothalamus; TU, tuberal nucleus; fx, fornix.
(B–D) Neuronal selectivities averaged across neurons that were recorded at the same site. Color indicates average neuronal selectivity.
(E–G) Neuronal selectivity as a function of the anteroposterior axis. Data represent mean ± SEM (n = 25, 12, 6, 24, 22 neurons from anterior to posterior).
Male selectivity (B and E), female selectivity (C and F), and spatial selectivity (D and G) are shown.
(G) Average information rate significantly changed along the anteroposterior axis (means with different letters are significantly different; $P < 0.05$, post hoc Tukey-Kramer test after significant one-way ANOVA).

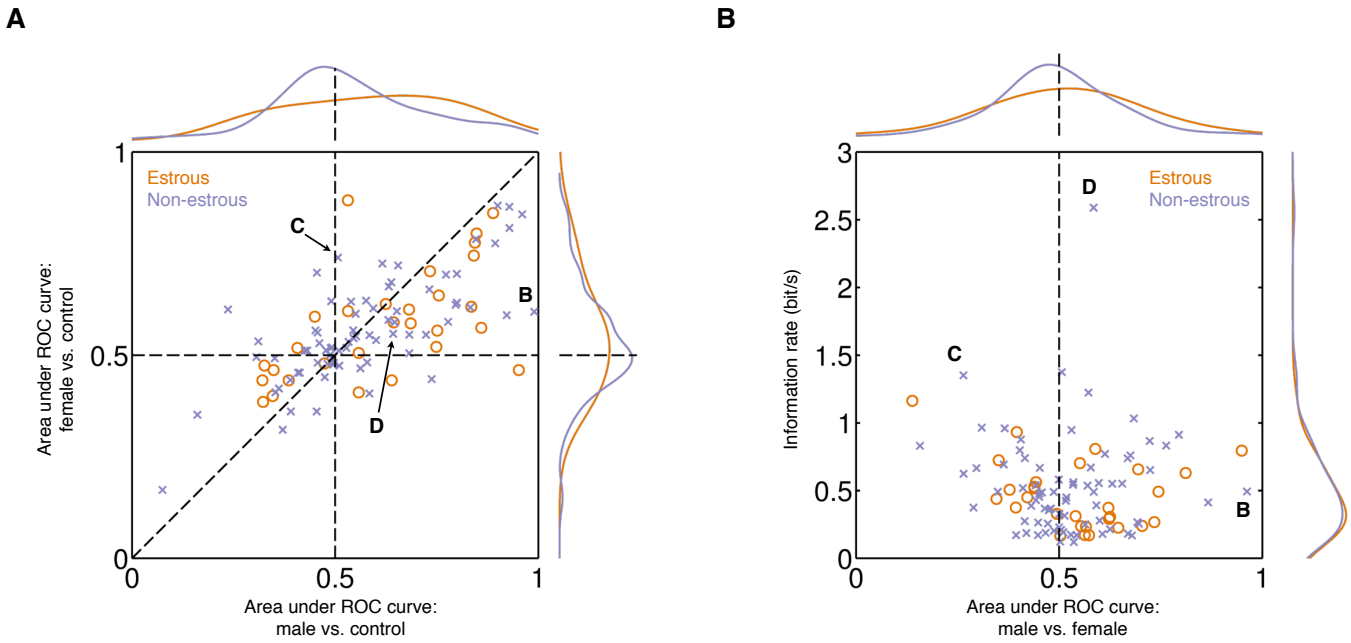

Figure S4. No Effect of the Reproductive State on Gender and Spatial Selectivity, Related to Figure 4.
The reproductive states are indicated by orange circles (estrous, n = 29 recordings) and blue crosses (non-estrous, n = 76 recordings).
(A) Gender selectivity. This figure results from re-examination of Figure 3E. The legends are the same as in Figure 3E.
(B) Spatial selectivity around the interaction partner. This figure results from re-examination of Figure 3F. The legends are the same as in Figure 3F.

Supplemental Experimental Procedures

Animals

All procedures were reviewed and performed in accordance with the Champalimaud Centre for the Unknown Ethics Committee guidelines, and approved by the Portuguese Veterinary General Board. Data was collected from sexually naive female C57BL/6 mice (n = 7; 2–6 months old). The animals were housed in groups under reversed light cycle conditions (12:12 light/dark cycle; light on 20h00). Food and water were available ad libitum. The estrous phase was determined by daily examination of vaginal lavage. Briefly, the proestrous phase was characterized by dominance of nucleated cells; the estrous phase was characterized by predominant cornified cells; the diestrous phase was characterized by the presence of leukocytes. Three estrous phases were grouped into two reproductive states: estrous (sexually receptive) and non-estrous (not receptive). The estrous state includes the proestrous phase, and the non-estrous state includes the estrous and diestrous phases. The results and conclusions were not qualitatively different when three reproductive states (i.e., proestrous, estrous, and diestrous) were used for analysis (data not shown). Only animals that showed regular estrous cycle were included for the following experiments. We used BALB/c mice as stimulus animals. FVB/N mice were also used in some experiments. Stimulus males were sexually experienced and housed in isolation, while stimulus females were sexually naive and housed in groups.

Surgical procedures

Mice were anesthetized with 2–4% isoflurane in oxygen and fixed in a stereotaxic frame using ear-bars (Kopf). During surgery, anesthesia was maintained with 0.5–2% isoflurane. A movable 16-channel electrode drive (two bundles of eight 35-μm tungsten wires; impedance, ~600 kOhms measured at 1,000 Hz; Innovative Neurophysiology) was inserted into the brain, and then fixed to the skull with dental acrylic. The target coordinates were 1.45 mm posterior to bregma, 0.70 mm lateral to the midline, and approximately 5.8 mm ventral from the brain surface. A stainless screw was inserted into the skull and served as ground. Analgesia was administered for at least 2 days. The animals implanted with the drive were singly housed for 2 days, then returned to their home cage, and allowed at least 7 days for post-operative recovery.

Behavioral paradigm

The experiments were performed under red dim light during the dark period in which mice are socially active. The subject female was connected with the cable for electrophysiological experiments under a brief isoflurane immobilization. The subject female was then moved to the apparatus (40 cm × 40 cm × 25 cm or 39 cm × 18 cm × 17 cm), and habituated for 30 minutes. A behavioral session consisted of several blocks (0.5–5 minutes): control, male, female, and object. No stimulus was presented during the control block. In order to control perturbation, the experimenter pretended to bring in and take away from the apparatus at the beginning and end of the block. The stimulus male and female was presented during the male and female block, respectively. The stimulus animal was introduced to the apparatus at the farthest location from the subject female. The subject female was allowed to freely interact with the stimulus animal, although the stimulus animal was removed if it showed excessive aggression or attempted to copulate. A plastic bottle cap was presented at the center of the apparatus during the object block. The data from the object block was similar to that from the female block (data not shown). The sessions were videotaped from the top (Stingray, AVT; frame rate, 50 Hz; synchronized to neural data acquisition; Cineplex, Plexon).

Electrophysiology

Multiple single-unit activities were recorded while the animal performed the behavioral paradigm. Neural signals were amplified (gain, 8,000×) and filtered (bandpass filter, 300–6,000 Hz) through a headstage and a differential pre-amplifier (Omniplex, Plexon). The electrode with no recorded unit served as a local reference. The signals were then digitized at 40 kHz. Waveforms were extracted online by thresholding the filtered signals and stored in the computer for the offline analysis. Neural activities were monitored by a software oscilloscope and a loudspeaker (A-M systems). The thresholded neural signals were sorted offline using a sorting software based on principal component analysis (Offline Sorter, Plexon). A cluster consisting of similar waveforms was defined manually in the feature space, and regarded as a single unit. The interspike intervals (ISIs) within one cluster should be greater than 1.2 ms. A cross-correlogram was examined to reject duplicate recordings from the same neuron.

If we recorded neurons that had similar waveforms in the same channel at the same recording depth for successive sessions (point-by-point Pearson's $r > 0.9$; a liberal threshold was chosen so that duplicate recordings from the same neuron were rejected [S1]), these neurons were regarded as the same neuron. If the same neuron was recorded for multiple times under a given reproductive state, we only used the data from the session in which this neuron was recorded for the first time. Therefore, one particular neuron could appear in the two reproductive states, but should not appear twice within a given reproductive state.

Analysis

All data was analyzed using Matlab (Mathworks) unless otherwise noted. Data was represented as mean ± SEM in the main text.

Behavioral analysis

Representative positions of the two animals (body center, nose, and tail base) were detected by a modified version of the custom-made software (https://github.com/joseaccruz/SimpleMouseTracker; using OpenCV library through Python). The orientation of each mouse was calculated from the coordinates of body center and nose. Moving distance, normalized by time, was calculated. Additionally, we computed four measures (frequency, duration, latency, and the percentage of time engaged) for three behaviors (approach, withdrawal, and interaction) which were annotated by the behavior annotating software, Janelia Automatic Animal Behavior Annotator (JAABA) [S2]. Briefly, the experimenter marks a behavior of interest while monitoring a video. Then, JAABA computes a classifier of the behavior by applying machine learning algorithm on automatically calculated video features. The experimenter can iteratively repeat this process until JAABA produces satisfactory results about behavioral annotations. Approach was marked when one animal moved towards another animal. Withdrawal was marked when one animal moved away from another animal. In the approach and withdrawal events, it was irrelevant what another animal was doing. Interaction was marked when two animals were close to each other. These measures were statistically tested by two-way ANOVA with the reproductive state of the subject female and the gender of the stimulus animal as the main effects.

To analyze the behavior of the subject female during social interactions, behavioral occupancy maps around the interaction partner were created in coordinates in which the stimulus animal was centered (Figure 1C). For each video frame, the image was rotated so that the stimulus animal oriented upwards, and the nose position of the subject female was marked. Since we were not interested in behavioral laterality in this study, the left side of the coordinates was merged onto the right side. These procedures were repeated for all video frames, resulting in a scatter plot that showed the nose positions of the subject female around the stimulus animal. A behavioral occupancy map was created by spatially binning this plot (3.64 cm × 3.64 cm). We ignore the bins in which the number of visits was less than 5. Head-tail preference index was defined as (h-t) / (h+t), where h and t indicate the probability of the subject female being in the two spatial bins around the head and tail of the stimulus animal, respectively.

Neuronal data analysis

The baseline firing rate was defined as the mean firing rate during the control block. For 16 neurons that were recorded in both states, we used the average firing rate between the two reproductive states. To understand how neuronal activity is modulated during social interactions, firing rate maps were computed by dividing the number of spike counts by the number of visits. The bins in which the number of visits was less than 5 were ignored. Firing maps were smoothed by a gaussian kernel with full width at half maximum of 8.55 cm.

A single neuron was considered responsive if the distribution of the ISIs during the stimulus block was significantly different from the ISI distribution during the control block ($P < 0.01$, two-tailed Kolmogorov-Smirnov test). Responsive neurons were further classified into two types by comparing the mean firing rate during the stimulus block with the mean firing rate during the control block: excited and inhibited types. When there were less than 10 ISIs during one block (3 out of 105 recordings, 2.9%), the neuronal type was determined by visual inspection.

To quantify gender selectivity, we performed receiver operating characteristic (ROC) analysis [S3, S4]. A block was divided into multiple one-second bins, and the number of spikes per bin was used as a sample in ROC analysis. The area under the ROC curve represents how accurate an ideal observer could tell the block condition from which a sample is drawn. We used the area under the ROC curve (A vs. B) as an index for gender selectivity with a range from 0 to 1, where 0 represents “preference to B,” 1 represents “preference to A,” and 0.5 represents no selectivity. If there were multiple blocks available for a given stimulus type, the maximum ROC value was used for the analysis.

To quantify spatial selectivity, information rate about the firing rate map was computed by the following equation [S5]. The information rate was computed for an individual firing map. The maximum information rate among those for male and female blocks was used for the analysis.

$$I = \sum_x \lambda(x) \log_2 \left(\frac{\lambda(x)}{\lambda} \right) p(x)$$

where I is the information rate in bits per second, x is spatial location (i.e., a particular bin in the firing map). $p(x)$ is the probability for the animal being at location x. $\lambda(x)$ is the mean firing rate when the animal is at location x. λ is the overall mean firing rate.

To statistically test the effect of the reproductive state on the firing rate, we performed a permutation test. The difference in the mean firing rate between the two reproductive states was used as a test statistics. The test statistics was calculated from the original dataset as well as from 10,000 randomized datasets in which the labels (i.e., reproductive state) were permuted. Statistical significance was determined by comparing the test statistics from the original dataset to the distribution of the test statistics obtained from randomized datasets. Two-tailed P value was calculated as the proportion of the test statistics from randomized datasets where the absolute value was greater than the absolute value of the test statistics from the original dataset.

Histology

After completion of the recording experiments, the recording sites were marked by passing currents (20 μA, 40 s; model A365, WPI). The animals were deeply anesthetized and perfused transcardially with cold 0.9% saline and 4% paraformaldehyde (PFA) in 1× PBS. The brains were removed from the skulls and post-fixed in the 4% PFA solution at 4°C overnight. The brains were cut in 50-μm-thick coronal sections with a vibratome (VT1000S, Leica). The sections were subjected to Nissl staining. The recording sites were reconstructed based on the positions of microlesions, the trace of the cannulas, and the recording depth.

Copulation experiments

In order to examine how social interactions between females and males would develop depending on the reproductive state in a longer timeframe, we performed separate behavioral experiments where copulation was allowed. Fourteen C57BL/6 sexually naive females and seven BALB/c sexually experienced males were used. Each male was paired with two females (one estrous and one non-estrous females; the order of presentation was counterbalanced). Two females were excluded from the analysis because the male that was paired with those females lacked sexual motivation. The female was introduced to the apparatus (40 cm × 40 cm × 25 cm), and habituated for 30 minutes. Then, the male was introduced, and the animals were allowed to interact freely. The experiment was finished when the male ejaculated or when 60 minutes had elapsed. One experiment was aborted at ~20 minutes because the female jumped out of the apparatus. The experiments were videotaped for offline analysis. Six behaviors were manually annotated [S6] (http://cowlog.org/; interaction, female approach, male approach, male mounting, male intromission, male ejaculation). Frequency and latency to the first event were computed for each behavior. In addition, mean duration and the percentage of time engaged were calculated for interaction and male intromission behaviors. Peri-event time histograms of these behavioral events were also examined (histograms were aligned either to the time of male entry or to the time of the first mounting or, if applicable, intromission). Lordosis quotient was defined as the ratio of the number of intromissions to the number of mounting attempts multiplied by 100. These behavioral measures were statistically tested by Mann-Whitney U test.

Supplemental References

S1. Jackson, A., and Fetz, E.E. (2007). Compact movable microwire array for long-term chronic unit recording in cerebral cortex of primates. J. Neurophysiol. *98*, 3109–3118.

S2. Kabra, M., Robie, A.A., Rivera-Alba, M., Branson, S., and Branson, K. (2013). JAABA: interactive machine learning for automatic annotation of animal behavior. Nat. Methods *10*, 64–67.

S3. Green, D.M., and Swets, J.A. (1996). Signal detection theory and psychophysics. (New York: Wiley).

S4. Feierstein, C.E., Quirk, M.C., Uchida, N., Sosulski, D.L., and Mainen, Z.F. (2006). Representation of spatial goals in rat orbitofrontal cortex. Neuron *51*, 495–507.

S5. Skaggs, W.E., McNaughton, B.L., Gothard, K., and Markus, E.J. (1993). An information-theoretic approach to deciphering the hippocampal code. In Advances in Neural Information Processing Systems 5, S.J. Hanson, J.D. Cowan, and C.L. Giles, eds. (San Mateo: Morgan Kaufmann), pp. 1030–1037.

S6. Hänninen, L., and Pastell, M. (2009). CowLog: Open source software for coding behaviors from digital video. Behav. Res. Methods *41*, 472–476.