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Supplementary information

Distinct hypothalamic control of same- and opposite-sex mounting behaviour in mice

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Supplementary Notes

SUPPLEMENTARY NOTE 1 – Related to Fig. 1b, c and Extended Data Fig. 1a-g

We trained two supervised classifiers (decoders) to distinguish female- and male-directed mounting, using the same set of videos, but different sets of frames to extract mouse pose features (**Supplementary Table 1**). One classifier was trained using pose features derived exclusively from frames in which actual mounting bouts occurred ("frame-by-frame decoder"; **Fig. 1b, c, ED Fig. 1b**). The other was trained using pose features extracted from video frames spanning 3 seconds before to 1 second after mount onset ("temporal decoder"; **ED Fig. 1e-g**). Performance was higher using the temporal decoder (78%) than the frame-by-frame decoder (63%). This result suggests that that pose features extracted from frames prior to the initiation of mounting make a large contribution to decoder performance.

We examined four pose features (inter-mouse distance, resident axis ratio, resident acceleration, and resident nose speed) which made a large contribution to distinguishing female- vs. male-directed mounting in the temporal decoder. Feature histograms (**ED Fig.1g**, bottom row) for female- vs. male-directed mounting were better separated for the temporal decoder, than for the frame-by-frame decoder (**ED Fig. 1b**).

Closer inspection of individual features revealed that inter-mouse distance during the pre-mounting period was larger with female than male intruders. This may reflect the tendency of resident males to engage in relatively longer periods of close investigation of male intruders before mount initiation, whereas they approached and quickly initiated mounting to female intruders (**ED Fig.1d**). A difference in resident axis ratio was also evident during residents' pre-mounting behaviours towards male vs. female intruders. With female intruders, the axis ratio increased immediately before mount initiation since residents walked over to females in a stretched posture and then mounted. With male intruders, by contrast, the axis ratio was smaller since residents remained close to intruders and investigated them, resulting in a more contracted body posture.

Other feature differences may reflect distinctions in the intruders' peri-mount behaviours. Male intruders attempted to walk away from the residents when they were being intensively investigated, causing residents to follow them around; this behaviour is reflected in a higher resident acceleration for male than for female intruders. Similarly, male intruders tended to initiate escape when mounted, whereas female intruders remained in place and were receptive to mounting. This difference is reflected in a higher resident nose speed (locomotion) for male than for female intruders, after mount initiation. Thus, examination of different feature distributions during male- versus female-directed mounting provides some insights into how these behaviours differ, and the reasons for the difference in decoder performance.

SUPPLEMENTARY NOTE 2 – Related to Extended Data Fig. 4

Although the majority of male-directed mounting events were USV^- (**Fig. 1g, k**), we observed rare cases of animals that exhibited USV^+ male-male mounting. These cases included resident males with relatively less sexual and social experience (**Fig. 1g**, Day 1, green), or with castrated male intruders (**Fig. 4k**, green). Of ten animals implanted for dual-site fiber photometry and used for analysis, two showed USV^+ mounting toward male as well as female intruders (**Extended Data Fig. 4**). While they are rare examples, they provide useful cases of "exceptions that prove the rule."

One sexually and socially experienced male mouse (no. 629) showed USV^+ mounting towards male as well as female intruders (**Extended Data Fig. 4a, b**), and no attack. Scaled neural activity in this animal during USV^+ mounting towards males was higher in $MPOA^{ESR1}$ than $VMHvl^{ESR1}$ neurons, similar to the typical activity pattern exhibited towards female intruders (**Extended Data Figs. 3m, 4a-**

c). In a triadic social encounter (i.e., two intruders, one male and one female), this mouse preferred the female over the male. This suggests that the lack of attack and USV^+ male-directed mounting exhibited by this mouse was not due to a failure to discriminate the sex of intruders, e.g., as in *TrpC2* mutant mice[14,45]. In another example, a sexually and socially naïve mouse (no. 634) initially exhibited USV^+ mounting towards male as well as female intruders (**Extended Data Fig. 4f-h**), and (as in the case of mouse no. 629) scaled activity was higher in $MPOA^{ESR1}$ than in $VMHvl^{ESR1}$ neurons during male-directed USV^+ mounting. After gaining social and sexual experience, however, this animal switched to USV^- mounting towards male intruders, and exhibited a male intruder-typical pattern of activity in $MPOA^{ESR1}$ versus $VMHvl^{ESR1}$ neurons (**Extended Data Figs. 3q, 4i-k**). Thus, in both cases, these mice exhibited a female intruder-typical pattern of activity in $MPOA^{ESR1}$ versus $VMHvl^{ESR1}$ neurons when they exhibited USV^+ mounting towards males. This suggests that the relative level of activity in these neurons is not simply representing intruder sex, but rather reflects intent or motivational state.

SUPPLEMENTARY NOTE 3 – Related to Extended Data Fig. 10q

In **Extended Data Figure. 10q**, we present a working hypothesis to reconcile the results of imaging experiments with the effects of functional manipulations of $ESR1^+$ neurons in MPOA and VMHvl. Here we describe each class of functional manipulation, together with the most parsimonious explanation for its behavioural effects, in terms of the physiological properties of neuronal subsets detected in our imaging experiments.

VMHvl:

GOF: Strong optogenetic activation of $ESR1^+$ neurons and chemogenetic activation of progesterone receptor (PGR) positive PGR neurons promote attack[11,12]①, while weak optogenetic activation of $ESR1^+$ neurons promotes mounting without USVs ② (**Fig. 4a-k**). Strong activation of $VMHvl^{ESR1/PGR}$ neurons also inhibits female-directed sexual behaviour[11], via likely projections to MPOA ③ (**Fig. 4o-r**). Since virtually all $VMHvl^{ESR1}$ neurons are glutamatergic[16], this inhibition likely recruits local inhibitory interneurons in MPOA ④, which is 80% GABAergic[15] (although this is not proven). The larger proportion of male- vs. female- selective $ESR1^+$ neurons in VMHvl (~2.4:1; ref[6] and this study) likely explains why aggressive behaviours dominate the response to GOF manipulations of $VMHvl^{ESR1/PGR}$ neurons[11,12].

LOF: Genetic ablation[10] or chemogenetic inhibition of $VMHvl^{PGR}$ neurons[11] weakly inhibits female-directed male mounting. Here we show that chemogenetic inhibition of $VMHvl^{ESR1}$ neurons strongly inhibits female-directed mounting (**ED Fig. 9j-m**), using a longer incubation time between CNO injection and testing. It is likely (but not directly demonstrated) that this effect is due to inhibition of the female-selective subset of neurons in VMHvl ⑤ (refs[6,16,26] and **Fig. 2f, g**). Such chemogenetic inhibition presumably also silences $VMHvl^{ESR1}$ neurons that inhibit mating via projections to MPOA ③ (**Fig. 4o-r**), which in theory should increase mating. However, these neurons are likely found in the male-preferring population, and therefore should not be active in the presence of a female; hence silencing them has no effect. The female-preferring $VMHvl^{ESR1}$ neurons that are required for sexual mounting may project to MPOA (not illustrated) or to a downstream target to which MPOA also projects.

MPOA:

GOF: Optogenetic activation of $MPOA^{ESR1}$ neurons evokes mounting very inefficiently[8] (**Fig. 3c, d**). This may be because the $ESR1^+$ population in MPOA is heterogeneous, and contains multiple subpopulations with different functions[15]; if these subpopulations interfere with each other, then their simultaneous activation may cancel each other out. Intersectional activation of GABAergic $ESR1^+$

neurons stimulates a more restricted population and therefore these competing effects are eliminated, yielding more efficient promotion of mounting ⑥ (**Fig. 3c, d, ED Fig. 7**). Activation of $MPOA^{ESR1\cap VGAT}$ neurons promotes both mounting towards intruders, and USVs in solitary males. Whether these are the same or different subsets of $MPOA^{ESR1\cap VGAT}$ neurons cannot be distinguished at present. Gao et al. recently reported that optogenetic stimulation of $MPOA^{VGAT}$ neurons promotes USVs[23], but whether these neurons were $ESR1^+$ was not determined. Stimulation of $MPOA^{ESR1\cap VGAT}$ terminals in VMHvl suppresses aggression ⑦ (**Fig. 4s, t**). Whether this occurs via collateral projections of the same MPOA neurons as promote mounting, or via a different subpopulation of MPOA neurons, is not yet clear.

One might predict that optogenetic activation of the male-selective neurons in MPOA would promote aggressive behaviour ⑧. However, male sexual behaviour dominates when $MPOA^{ESR1\cap VGAT}$ neurons are optogenetically stimulated, because the female-selective neurons outnumber the male-selective neurons in MPOA by ~2:1 (**Fig. 2f, g**).

LOF: Silencing $MPOA^{ESR1}$ neurons reduces both mounting[7] and USVs[9] ⑥ (**Fig. 2m-o, ED Fig. 9c-e**). There is no effect on aggressive behaviour (**ED Fig. 9g-i**), perhaps because such silencing also inactivates MPOA neurons that normally inhibit aggression via projections to VMHvl ⑦, thereby increasing VMHvl activity. In rare cases, we have observed that silencing MPOA releases aggression towards females.

Single-cell RNA sequencing has identified 6-7 transcriptomically distinct subsets of $ESR1^+$ neurons in VMHvl[16]. A similar diversity of $ESR1^+$ neurons has been identified in the POA[15]. Given that binary intersectional techniques were required to selectively activate $ESR1^+$, $VGAT^+$ neurons in MPOA that promote sexual behaviour (this study), it is likely that triple or even higher-order genetic intersectional techniques will be required to functionally isolate smaller subsets of these cells. Such techniques are currently challenging in mice, but may become more feasible in the future.

45. Leypold, B. G. et al. Altered sexual and social behaviors in trp2 mutant mice. *Proc. Natl Acad. Sci. USA* **99**, 6376–6381 (2002).