Title: Short-term social isolation acts on hypothalamic neurons to promote social
behavior in a sex- and context-dependent manner.

Xin Zhao[1], Yurim Chae[1], Destiny Smith[1], Valerie Chen[1], Dylan DeFelipe[1], Joshua W Sokol[1],
Archana Sadangi[1], and Katherine Tschida[1]

[1]Department of Psychology, Cornell University, Ithaca NY 14853

*Corresponding Author: Katherine Tschida, kat227@cornell.edu

Abstract

Social animals, including both humans and mice, are highly motivated to engage in
social interactions. Short-term social isolation increases social motivation and promotes
social behavior, but the neural circuits through which it does so remain incompletely
understood. Here, we sought to identify neurons that promote social behavior in single-
housed female mice, which exhibit increased rates of social investigation, social ultrasonic
vocalizations (USVs), and mounting during same-sex interactions that follow a period of
short-term (3-day) isolation. We first used immunostaining for the immediate early gene Fos
to identify a population of neurons in the preoptic hypothalamus (POA) that increase their
activity in single-housed females following same-sex interactions (POA_{iso} neurons). TRAP2-
mediated chemogenetic silencing of POA_{iso} neurons in single-housed females significantly
attenuates the effects of short-term isolation on social investigation and USV production and
also tends to reduce mounting. In contrast, caspase-mediated ablation of POA_{iso} neurons in
single-housed females robustly attenuates mounting but has no effect on social investigation
or USV production. Optogenetic activation of POA_{iso} neurons in group-housed females
promotes USV production but does not recapitulate the effects of short-term isolation on
social investigation and mounting. To understand whether a similar population of POA_{iso}
neurons promotes social behavior in single-housed males, we performed Fos
immunostaining in single-housed males following either same-sex or opposite-sex social
interactions. These experiments revealed a population of POA neurons that increase Fos
expression in single-housed males following opposite-sex, but not same-sex, interactions.
Chemogenetic silencing of POA_{iso} neurons in single-housed males during interactions with
females tends to reduce mounting but does not decrease social investigation or USV
production. These experiments identify a population of hypothalamic neurons that promote
social behavior following short-term isolation in a sex- and social context-dependent manner.

Keywords: social isolation, ultrasonic vocalization, preoptic hypothalamus, mounting,
female

Introduction

Humans and other social mammals find social interactions rewarding and are highly
motivated to seek out social connections. Consequently, the experience of social isolation is
aversive and impacts both our brains and our behaviors. While long-term isolation can lead
to the emergence of anti-social behaviors in both humans and rodents (An et al., 2017;
Arrigo and Bullock, 2008; Check et al., 1985; Hossain et al., 2020; Killgore et al., 2021; Ma
et al., 2011, 2022; Machimbarrena et al., 2019; Matsumoto et al., 2005; Mears and Bales,
2009; Reid et al., 2022; Toth et al., 2011; Valzelli, 1973; Weiss et al., 2004; Wiberg and
Grice, 1963; Zelikowsky et al., 2018), short-term isolation typically increases levels of social
motivation and promotes social-seeking behaviors (Baumeister and Leary, 1995; Cacioppo
et al., 2006; Cacioppo and Cacioppo, 2018; House et al., 1988; Lee et al., 2021; Niesink and
van Ree, 1982; Panksepp and Beatty, 1980; Zhao et al., 2021). Alterations in social
motivation are characteristic of many neurodevelopmental disorders, including autism
spectrum disorder (Chevallier et al., 2012; Clements et al., 2018). How short-term social
isolation acts on the brain to promote social behavior remains incompletely understood.

Mesolimbic circuits play an important role in regulating social motivation and social
reward, during courtship as well as during same-sex interactions (Bariselli et al., 2018; Dai et
al., 2022; Dölen et al., 2013; Gunaydin et al., 2014; Hung et al., 2017; Love, 2014; Melis et
al., 2022; Resendez et al., 2020; Robinson et al., 2011; Solié et al., 2022; Tang et al., 2014;
Walum and Young, 2018; Xiao et al., 2017). In line with their role in regulating social
behavior, changes in the function of mesolimbic circuits have been reported following long-
term (weeks-long) social isolation and/or early-life social isolation (McWain et al., 2022;
Musardo et al., 2022; Tan et al., 2021; Yorgason et al., 2016). The neural circuit changes
that mediate the effects of short-term isolation on social motivation in adult animals are
comparatively less explored, but here as well, recent studies in both humans and rodents
have implicated changes in various populations of midbrain dopamine neurons (Inagaki et
al., 2016; Matthews et al., 2016; Tomova et al., 2020). Beyond its effects on mesolimbic
circuits, whether social isolation acts on additional neuronal populations to promote social
interaction is unknown.

In recent work, we found that short-term (3-day) social isolation exerts robust effects
on the social behaviors of C57BL/6J female mice (Zhao et al., 2021). Relative to group-
housed females, single-housed females that subsequently engaged in same-sex interactions
exhibited increased rates of social investigation, increased rates of USVs, and were also
observed to mount female social partners, a behavior never observed in pairs of group-

housed females (Zhao et al., 2021). The robust effect of short-term isolation on these three
aspects of female social behavior provides a powerful paradigm to identify neurobiological
changes that mediate the effects of short-term isolation on social behavior. In the current
study, we combined this behavioral paradigm with Fos immunostaining and the TRAP2
activity-dependent labeling approach (Allen et al., 2017; DeNardo et al., 2019) to identify and
characterize a population of neurons in the preoptic hypothalamus that increase their activity
in single-housed females following same-sex social interactions (i.e., POA_{iso} neurons). We
next asked whether silencing or ablation of POA_{iso} neurons attenuates the effects of short-
term isolation on female social behavior, and whether artificial activation of POA_{iso} neurons
in group-housed females mimics the effects of short-term isolation on female social
behavior. Finally, we extended a subset of these experiments to single-housed males
engaged in opposite-sex and same-sex interactions, to understand whether short-term
isolation acts on the POA to promote social behavior in a manner that depends on either sex
or social context. This study identifies novel neurobiological mechanisms through which
short-term social isolation acts on the brain to promote social interaction. Our findings also
add to an emerging literature indicating that the POA regulates not only sexual behavior but
also female social behavior during same-sex interactions.

Results

Neurons in the preoptic hypothalamus increase their activity in socially isolated
female mice following same-sex social interactions

To identify changes in neuronal activity that may underlie the effects of short-term
isolation on female social behavior, we performed immunostaining for the immediate early
gene Fos in brain sections collected from group-housed and single-housed (3-days) subject
females following 30-minute social encounters in their home cages with a novel, group-
housed visitor female (Fig. 1A). In line with our previous behavioral findings (Zhao et al.,
2021), we observed that single-housed female residents spent more time investigating
visitors (Fig. 1B; Mann-Whitney U test, $p = 0.001$) and in many trials mounted visitors, a
behavior that was not observed in group-housed residents (Fig. 1C; 0 of 12 group-housed
residents and 10 of 13 single-housed residents mounted visitors; Mann-Whitney U test for
difference in total mounting duration, $p < 0.001$; see Table S1 for complete statistical
details). Female pairs that contained a single-housed resident also produced higher rates of
ultrasonic vocalizations (USVs) than pairs with a group-housed resident (Fig. 1D; Mann-
Whitney U test, $p < 0.001$). Although either female in a dyad can produce USVs (Warren et
al., 2020), the robust effects of short-term isolation on the non-vocal social behaviors of
single-housed females suggest that at least some of the elevation in USV rates is driven by

increased USV production by the single-housed resident. Given the robust effects of short-
term isolation on these three aspects of female social behavior, we focused our analyses on
two hypothalamic regions implicated in regulating these behaviors: the preoptic area (POA),
which regulates social approach (McHenry et al., 2017), social reward (Hu et al., 2021),
mounting (Floody, 1989; Karigo et al., 2021; Wei et al., 2018) and USV production (Chen et
al., 2021; Gao et al., 2019; Green et al., 2018; Karigo et al., 2021; Michael et al., 2020); and
the ventromedial hypothalamus (VMH), which regulates mounting (Hashikawa et al., 2017;
Karigo et al., 2021; Lee et al., 2014; Liu et al., 2022). We also examined Fos expression
within the caudolateral periaqueductal gray (PAG), based on the well-established role of this
region in the control of vocalization in vertebrates and USV production in mice (Chen et al.,
2021; Jürgens, 1994; Michael et al., 2020; Tschida et al., 2019). To test whether any
observed differences in Fos expression in these three regions were associated with
isolation-induced changes in social behavior rather than baseline differences between
groups, we also measured Fos expression in the POA, the VMH, and the PAG of group-
housed and single-housed females that did not engage in social interaction with novel
female visitors (Fig. 1E-F; Fig. S1).
These analyses revealed that baseline levels of Fos expression within the POA and
the VMH did not differ between group-housed and single-housed females (Fig. 1F, left and
middle, open bars; two-way ANOVA to analyze Fos expression within each brain region,
factor 1 = housing status, factor 2 = social interaction, followed by post-hoc Tukey's HSD
tests). Following social interactions with novel female visitors, single-housed females
exhibited robust increases in Fos expression within the POA (Fig. 1F, left; p < 0.001) but not
within the VMH (Fig. 1F, middle; p > 0.05). In contrast, Fos expression within these two brain
areas did not increase significantly in group-housed females that interacted with novel
female visitors (Fig. 1F; p > 0.05 for both comparisons). Similar to the POA, baseline Fos
expression within the PAG did not differ between group-housed and single-housed females
(p > 0.05), and only single-housed females displayed increased PAG Fos expression
following social interactions with novel female visitors (Fig. 1F, right; p < 0.001), a finding
that further supports the idea that single-housed females increase USV production during
same-sex interactions. POA Fos expression was significantly and positively correlated with
the total amount of time spent in resident-initiated investigation for both group-housed and
single-housed females (Fig. S1C, left; linear regression, p < 0.05), as well as with the total
number of mounting bouts produced by single-housed resident females (Fig. S1C, middle; p
< 0.01). In both group-housed and single-housed female residents, Fos expression tended
to correlate positively with total USVs, but these relationships were not significant (Fig. S1C,
right; p > 0.05 for both comparisons; see Fig. S1D-E for relationships of VMH Fos and PAG
Fos to vocal and non-vocal social behaviors). In summary, POA Fos expression increases

selectively in single-housed females following social interactions, and levels of POA Fos

expression are also well related to the production of specific types of social behaviors by

single-housed females.

To ask whether the effects of short-term isolation on female social behavior and POA

Fos expression are long-lasting, we measured social behaviors of female residents at three

timepoints: (1) on day 0, when female subjects were still group-housed; (2) on day 3, after

female subjects had been single-housed for 3 days; and (3) on day 17, after half of the

subject females had been re-group-housed with their same-sex siblings for two weeks and

the other half of the subject females remained single-housed for two weeks (Figs. 1G-I).

Brains of re-group-housed and 14-day single-housed subject females were collected after

the day 17 social interaction, and Fos expression within the POA was examined (Fig. 1K).

Consistent with our earlier findings, rates of social investigation and USV production

significantly increased following 3 days of social isolation (Fig. 1G, I; one-way ANOVAs with

repeated measures; $p < 0.05$ for day 0 vs. day 3 in both groups for both behaviors).

Following re-group-housing, time spent in social investigation and rates of USV production

tended to decrease to pre-isolation levels (Fig. 1G, I, top plots; $p = 0.06$ for day 0 vs. day 17

investigation time and day 0 vs. day 17 total USVs in re-group-housed females). In contrast,

females that were single-housed for 14 days continued to spend increased time in social

investigation (Fig. 1G, bottom plot; $p < 0.05$ for day 0 vs. day 3 investigation and for day 0

vs. day 17 investigation), and pairs containing 14-day single-housed residents continued to

produce elevated rates of USVs (Fig. 1I, bottom plot; $p < 0.05$ for day 0 vs. day 3 USVs and

for day 0 vs. day 17 USVs). Time spent mounting tended to follow the same trends as rates

of social investigation and USV production in re-group-housed and 14-day single-housed

females (Fig. 1H). Along with the attenuation of female social behaviors following re-group-

housing, we also found that POA Fos expression was significantly lower in re-group-housed

females relative to 14-day single-housed females (Fig. 1K; t-test, $p < 0.001$). These findings

support the idea that changes in female social behavior following short-term isolation are

reversible and are accompanied by decreased POA Fos expression. Hereinafter, we refer to

the population of POA neurons that increase Fos expression in single-housed females that

have engaged in same-sex interactions as POA_{iso} neurons, and we next conducted

experiments to test whether functional manipulations of POA_{iso} neuronal activity impact the

effects of short-term isolation on female social behavior.

Chemogenetic inhibition of POA_{iso} neurons attenuates the effects of social isolation

on female social investigation and USV production

If increased activity of POA_{iso} neurons contributes to the effects of short-term

isolation on female social behavior, one prediction is that reducing the activity of POA_{iso}

neurons in single-housed females will attenuate the effects of isolation on female social

behavior. To test this idea, we employed the TRAP2 activity-dependent labeling strategy to

chemogenetically silence POA$_{iso}$ neurons in single-housed females during social interactions

with novel, group-housed female visitors (Fig. 2A). Briefly, the POA of TRAP2 female mice

was injected bilaterally with a virus driving the Cre-dependent expression of the inhibitory

DREADDs receptor hM4Di. Three weeks later, females were single-housed for 3 days and

then given a 30-minute social encounter with a novel, group-housed female visitor in their

home cage. Following the social interaction, resident females were given an I.P. injection of

4-hydroxytamoxifen (4-OHT), which drives the transient expression of Cre recombinase in

recently active neurons and thereby enables the expression of hM4Di in POA$_{iso}$ neurons.

Subject females remained single-housed for an additional 24 hours and then were re-group-

housed with siblings for 2 weeks. Subject females were then single-housed a second time

for 3 days and subsequently given a 30-minute same-sex interaction following I.P. injection

of either saline (control) or clozapine-n-oxide (CNO) (saline and CNO tests were run 3 days

apart, and the order was counterbalanced across experiments).

Comparison of the social behaviors of single-housed females between CNO and

saline sessions revealed that chemogenetic silencing of POA$_{iso}$ neurons significantly

reduced resident-initiated investigation (Fig. 2B; N = 12; red points; two-way ANOVA with

repeated measures on one factor; $p < 0.01$). Inhibition of POA$_{iso}$ neurons also tended to

reduce mounting, although this effect was not statistically significant (Fig. 2D; Kruskal Wallis

test performed on difference in mounting time (CNO-saline) for each group; $p > 0.05$).

Finally, inhibition of POA$_{iso}$ neurons significantly reduced USV production (Fig. 2D; two-way

ANOVA with repeated measures on one factor; $p < 0.01$). In contrast, CNO treatment did not

affect the production of any of these social behaviors in single-housed females with GFP

expressed in POA$_{iso}$ neurons (Fig. 2B-D; N = 14; black points; $p > 0.05$ for all CNO vs. saline

comparisons in the POA$_{iso}$ FLEX-GFP control group). To investigate the specificity of these

effects to chemogenetic silencing of POA$_{iso}$ neurons, we also performed control experiments

in which activity-dependent chemogenetic silencing was performed caudal to the POA within

the anterior hypothalamus (AH) (Figs. 2B-D; N = 12; brown points) or within the VMH (Fig.

2B-D; N = 5; gray points). No significant effects of CNO treatment on resident-initiated

investigation, mounting, or USV rate were observed in these control groups (Figs. 2C-D; $p >$

0.05 for all). The effect of chemogenetic inhibition of POA$_{iso}$ neurons to decrease female

social behavior also cannot be attributed to an overall decrease in movement (Fig. 2E; $p >$

0.5 for difference in movement between saline and CNO sessions; see Methods). In

summary, we demonstrate that chemogenetic silencing of POA$_{iso}$ neurons attenuates

isolation-induced changes in social behavior in female mice.

Ablation of POA$_{iso}$ neurons attenuates the effects of social isolation on female mounting

In previous work investigating the role of the POA in regulating rodent social behaviors, studies have reported different effects on behaviors according to whether they employed reversible or irreversible neuronal silencing strategies. Studies that used chemogenetic or optogenetic methods to reversibly silence genetically-defined subsets of POA neurons report decreases in both USV production in males (Chen et al., 2021; Karigo et al., 2021) and in mounting in males and females during interactions with female social partners (Gao et al., 2019; Karigo et al., 2021). In contrast, studies employing caspase-mediated ablation of genetically-defined subsets of POA neurons (Gao et al., 2019; Wei et al., 2018) or electrolytic lesions of the POA (Bean et al., 1981) report decreased mounting but no effects on rates of USV production. To test whether permanent ablation of POA$_{iso}$ neurons attenuates the effects of social isolation on female behavior in a manner similar to the effects of chemogenetic inhibition, we used the TRAP2 activity-dependent labeling strategy to express caspase in and to thereby ablate POA$_{iso}$ neurons (Fig. 3A; see Methods). Vocal and non-vocal social behaviors of resident females were compared pre- and post-ablation, and the same measurements were made in control females expressing GFP in POA$_{iso}$ neurons.

In contrast to the effects of chemogenetic inhibition of POA$_{iso}$ neurons, we found that caspase-mediated ablation of POA$_{iso}$ neurons did not affect rates of social investigation in single-housed females, although both experimental and control females spent more time investigating visitors in the post-4-OHT session (Fig. 3B; two-way ANOVA with repeated measures on one factor; $p > 0.05$ for main effect of group, $p < 0.01$ for main effect of time, $p > 0.05$ for interaction effect). Ablation of POA$_{iso}$ neurons also failed to reduce USV production in pairs containing single-housed females (Fig. 3D; two-way ANOVA with repeated measures on one factor; $p > 0.05$ for pre-4-OHT vs. post-4-OHT USV rates in POA$_{iso}$-caspase females). Notably, ablation of POA$_{iso}$ neurons significantly reduced mounting in single-housed females (Fig. 3C; Mann Whitney U test performed on the difference in mounting time (post-4-OHT - pre-4-OHT, $p = 0.01$). Taken together with our chemogenetic inhibition data, these results show that both reversible inhibition or irreversible ablation of POA$_{iso}$ neurons in single-housed female mice attenuates the effects of short-term isolation on mounting behavior, whereas only chemogenetic inhibition of POA$_{iso}$ neurons attenuates the effects of short-term isolation on female social investigation and USV production.

Optogenetic activation of POA$_{iso}$ neurons elicits USV production

To understand whether artificial activation of POA_{iso} neurons can recapitulate the

effects of short-term isolation on female social behavior, we assessed the effects of

optogenetic activation of POA_{iso} neurons on the social behaviors of group-housed females.

The TRAP2 strategy was used to express either channelrhodopsin (ChR2) or GFP in POA_{iso}

neurons (Fig. 4A; see Methods), and females were re-group-housed for two weeks before

beginning optogenetic activation experiments. The effects of optogenetically activating

POA_{iso} neurons were first assessed for each subject female in a 5-minute solo session, in

which the female was tested alone in a behavior chamber while pulses of blue light were

delivered unilaterally to the POA (473 nm, 10 mW, 20-50 Hz, 10-20 ms pulses, 5-10s train

durations). The effects of optogenetically activating POA_{iso} neurons were then assessed for

each subject female in a 20-minute social session, in which a novel, group-housed female

visitor was added to the behavior chamber. The pair was allowed to interact in the absence

of optogenetic stimulation for the first and last 5 minutes of the social session, and pulses of

blue light were delivered to the POA of the subject female throughout the middle 10 minutes

of the session (Fig. 4A).

When POA_{iso}-ChR2 females were tested alone, we found that optogenetic activation

of POA_{iso} neurons elicited weak-to-moderate USV production in 4 of 8 females, but the

comparison of USV rates from pre-laser baseline to the laser stimulation period was not

significant at the level of the entire group (Fig. 4B; Mann Whitney U test performed on the

difference in USV rates (laser - pre-laser), $p = 0.09$). In POA_{iso}-GFP control females, laser

stimulation failed to elicit USV production (0 ± 0 USVs elicited in N = 6 POA_{iso}-GFP controls).

Interestingly, we found that when laser stimulation was applied during social sessions,

optogenetic activation of POA_{iso} neurons more readily elicited USV production than in solo

sessions (Fig. 4C; USVs elicited by blue laser stimulation in 7 of 8 POA_{iso}-ChR2 females;

Mann Whitney U test performed on the difference in USV rates (laser - pre-laser), $p =$

0.006). Moreover, optogenetic activation elicited higher rates of USVs when applied at times

when subject females were in close proximity to visitor females (within 2 mouse body

lengths) as compared to times when the females were farther apart (mean increase in USV

rates from pre-laser to laser period was 2.96 ± 2.32 USVs/s for "near" stimulations, $1.84 \pm$

1.75 USVs/s for "far" stimulations; paired t-test performed on the difference in USV rates

(laser - pre-laser) for "far" vs. "near" stimulations; $p = 0.02$). In summary, optogenetic

activation of POA_{iso} neurons elicits USV production in group-housed females, and the

efficacy of this effect is modulated by social context and proximity to a social partner.

In contrast to the effects on USV production, optogenetic activation of POA_{iso}

neurons failed to extend the duration of social investigation bouts (paired t-test performed on

mean duration of social investigation bouts for each POA_{iso}-ChR2 female that overlapped

with laser stimulation vs. those that did not; $p > 0.05$). Moreover, optogenetic activation of

POA$_{iso}$ neurons only infrequently elicited mounting (activation elicited n = 1 bout of mounting

in N = 1 POA$_{iso}$-ChR2 female, n = 2 bouts of mounting in N = 1 POA$_{iso}$-ChR2 female, and n =

0 bouts of mounting in the remaining N = 7 POA$_{iso}$-ChR2 females). In summary, optogenetic

activation of POA$_{iso}$ neurons elicits USV production from group-housed females, particularly

when female subjects are engaged in interactions with female visitors, but otherwise fails to

recapitulate the effects of short-term isolation on the social behaviors of female mice.

Previous studies have found that USV production can be elicited in female and male

mice by artificial activation of VGAT$^+$ POA neurons (Gao et al., 2019), Esr1$^+$ POA neurons

(which are predominantly VGAT$^+$) (Chen et al., 2021; Michael et al., 2020), as well as POA

neurons that send axonal projections to the caudolateral PAG (which are predominantly

VGAT$^+$) (Chen et al., 2021; Michael et al., 2020). To ask to what extent POA$_{iso}$ neurons

overlap with these previously described populations, we first evaluated the neurotransmitter

phenotype of POA$_{iso}$ neurons by performing two-color in situ hybridization for c-fos mRNA

and vesicular GABA transporter (VGAT) mRNA and calculating the percentage of Fos$^+$ POA

neurons that co-expressed VGAT. This analysis revealed that a majority of POA$_{iso}$ neurons

are GABAergic (Fig. S2A-B; N = 4, 76 ± 8.8%). We next used the TRAP2 activity-dependent

labeling strategy to express GFP in POA$_{iso}$ neurons and found GFP-positive axons within the

caudolateral PAG, indicating that at least some POA$_{iso}$ neurons send axonal projections to

the PAG (Fig. S2C-D; see Methods). Finally, we combined retrograde tracing from the

caudolateral PAG with Fos immunostaining to quantify the percentage of PAG-projecting

POA neurons that increase Fos expression in single-housed females following same-sex

interactions. This experiment revealed that around 20% of PAG-projecting POA neurons

express Fos in single-housed females following same-sex interactions (Fig. S2E-F; N = 4

females, percentage of tdTomato neurons that are Fos-positive = 18.3 ± 2.9%). These

findings suggest that a subset of POA$_{iso}$ neurons overlap with GABAergic, PAG-projecting

POA neurons that have been demonstrated in previous work to promote USVs via

disinhibition of excitatory PAG neurons important to USV production (Chen et al., 2021;

Michael et al., 2020).

312 POA neurons increase their activity in single-housed male mice following opposite-

313 sex but not same-sex social interactions

Given our findings that POA$_{iso}$ neurons contribute to isolation-induced changes in the

social behaviors of female mice, we next wondered whether a similar population of POA

neurons contributes to isolation-induced changes in social behavior in male mice. To

address these questions, we measured the vocal and non-vocal social behaviors of sexually

naïve males, which were either group-housed with same-sex siblings or single-housed for

three days and then given a 30-minute social interaction with a novel, group-housed visitor.

To consider the effects of isolation on male social behavior in different social contexts, males

were given either a social encounter with a same-sex visitor (MM context) or with an

opposite-sex visitor (MF context). Following these social interaction tests, we collected the

brains of the subject males and performed immunostaining to measure Fos expression

within the POA. Consistent with our prior work (Zhao et al., 2021), we found that males

exhibit higher rates of social investigation, higher rates of mounting, and produce more

USVs during interactions with females than during same-sex interactions (Fig. 5A-C; $p <$

0.05 for main effect of social context for all three behaviors). With respect to resident-

initiated investigation, we found a significant main effect of housing, indicating that single-

housed males spent more time investigating visitors during both opposite-sex and same-sex

interactions (Fig. 5A; two-way ANOVA, $p = 0.02$ for main effect of housing). In contrast,

single-housed males spent more time mounting female visitors than did group-housed

males, but there were no differences in mounting between single-housed and group-housed

males during same-sex interactions (Fig. 5B; two-way ANOVA, $p < 0.05$ for difference

between single-housed males interacting with females and all other groups). Similarly, there

was also a context-dependent effect of social isolation on male USV production, whereby

only single-housed males that interacted with female visitors exhibited increased USV

production relative to group-housed males (Fig. 5C; two-way ANOVA with post-hoc Tukey's

HSD tests; $p < 0.001$ for total USVs in single-housed MF vs. group-housed MF trials; $p >$

0.05 for total USVs in single-housed MM vs. group-housed MM trials). The finding that short-

term isolation exerts larger effects on male social behavior during subsequent opposite-sex

interactions relative to same-sex interactions is consistent with prior work (Zhao et al., 2021).

When we examined POA Fos expression in these four groups of males, we found that POA

Fos was significantly elevated in single-housed males following interactions with females

relative to the other three groups (Fig. 5D; two-way ANOVA, Tukey's post-hoc HSD tests; p

< 0.05 for difference in POA Fos between single-housed MF and all other groups). In

summary, the effects of short-term isolation on male social behavior are context-dependent,

and increased Fos expression within the POA is seen in single-housed males following

interactions with females, a context marked by increased male social investigation,

increased male mounting, and increased male USV production.

To test whether neural activity in male POA_{iso} neurons contributes to isolation-

induced changes in male social behavior, we used the TRAP2 strategy to chemogenetically

silence POA_{iso} neurons in single-housed males during social interactions with novel, group-

housed females (see Methods). The vocal and non-vocal behaviors of subject males were

measured and compared during 30-minute social interactions following I.P. injection of either

saline or CNO. Control males were treated identically but were injected with a virus to drive

expression of GFP in POA$_{iso}$ neurons. In contrast to our findings in females, chemogenetic

inhibition of male POA$_{iso}$ neurons tended to reduce time spent mounting (Fig. 5G; Mann

Whitney U test performed on difference in mounting time (CNO - saline); $p = 0.13$ for

difference between groups) but did not change rates of resident-initiated social investigation

(Fig. 5F; two-way ANOVA with repeated measures on one factor; $p = 0.01$ for main effect of

group; $p > 0.05$ for main effect of drug and for interaction effect) and also did not affect USV

rates (Fig. 5H; two-way ANOVA with repeated measures on one factor; $p > 0.05$ for main

effects and interaction effect). In summary, we find that chemogenetic silencing of male

POA$_{iso}$ neurons tends to reduce mounting during subsequent social interactions with females

but does not reduce social investigation or USV production, a pattern of results that differs

from the effects on single-housed female social behavior of chemogenetically silencing

female POA$_{iso}$ neurons.

Discussion

In the current study, we identify and characterize a population of preoptic

hypothalamic neurons that contribute to the effects of short-term social isolation on the

social behaviors of mice. These POA$_{iso}$ neurons exhibit increased Fos expression in single-

housed female mice following same-sex social interactions, and this increase in Fos

expression scales positively with the time females spend investigating and mounting female

visitors and tends also to scale with rates of USVs. Chemogenetic silencing of POA$_{iso}$

neurons attenuates the effects of social isolation on female social behavior, significantly

reducing social investigation and USV production while tending to reduce mounting. In

contrast, irreversible ablation of POA$_{iso}$ neurons significantly reduces mounting in single-

housed females but has no effect on rates of social investigation or USVs. Optogenetic

activation of POA$_{iso}$ neurons partially recapitulates the effects of short-term isolation on

female behavior and promotes USV production in female mice, particularly during same-sex

social interactions and when in close proximity to female visitors. Finally, we extended our

analyses to male mice to understand whether similar POA neurons may mediate changes in

male social behavior following short-term isolation. We find that short-term isolation exerts

more robust effects on male behavior during subsequent interactions with females than

during subsequent interactions with males, and increased POA Fos expression is only seen

in single-housed males following social interactions with females. Interestingly,

chemogenetic silencing of these POA$_{iso}$ neurons tends to reduce mounting but has no effect

on social investigation and USV production, in contrast to the effects of chemogenetically

silencing POA$_{iso}$ neurons in females. Together, these experiments identify a population of

preoptic hypothalamic neurons that promote social behaviors in single-housed mice in a

manner that depends on sex and social context.

392   An extensive body of past work has implicated the POA in the regulation of male

sexual behavior, including in the regulation of male courtship vocalizations in both rodents

and birds (Alger and Riters, 2006; Bean et al., 1981; Gao et al., 2019; Merari and Ginton,

1975; Riters, 2012; Riters et al., 2000; Riters and Ball, 1999; Wei et al., 2018). Past work

has also shown that activation of genetically-defined subsets of POA can elicit the

production of USVs in both male and female mice (Chen et al., 2021; Gao et al., 2019;

Karigo et al., 2021; Michael et al., 2020). However, whether the POA regulates natural USV

production in female mice remained unclear. In the current study, we demonstrate that

reversible silencing of POA neurons that increase their activity during same-sex interactions

decreases female USV production, indicating that the POA regulates the production of USVs

by single-housed females engaged in same-sex interactions. Whether these same neurons

regulate female USV production in other behavioral contexts, including in group-housed

females and in females interacting with male partners, remains an important open question.

In addition to attenuating USV production, we found that silencing of POA_{iso} neurons in

single-housed females significantly reduced social investigation of a same-sex partner and

also tended to reduce mounting. Our retrograde and anterograde tracing experiments

demonstrate that at least a subset of POA_{iso} neurons project to the caudolateral PAG where

neurons important for USV production reside, and an attractive possibility is that POA_{iso}

neurons promote USV production via disinhibition of PAG-USV neurons as previously

demonstrated for genetically-defined subsets of POA neurons (Chen et al., 2021; Michael et

al., 2020; Tschida et al., 2019). Future experiments will be required to determine whether

PAG-projecting POA_{iso} neurons also regulate social investigation and mounting, or

alternatively, whether distinct molecularly-defined or projection-defined subsets of POA_{iso}

neurons differentially regulate these different aspects of female social behavior. The latter

organization would be reminiscent of how projection-defined subsets of galanin-expressing

POA neurons regulate different aspects of parental behavior (Kohl et al., 2018).

418   Although reversible inhibition of POA_{iso} neurons reduced both social investigation and

USV production in single-housed females, permanent caspase-mediated ablation of these

neurons significantly reduced mounting but had no effects on social investigation or USV

production. These differences are largely consistent with prior studies that reported effects

on both mounting and USV production following reversible manipulations of POA activity and

effects on mounting but not on USV production following irreversible manipulations of POA

activity (Bean et al., 1981; Chen et al., 2021; Gao et al., 2019; Karigo et al., 2021; Wei et al.,

2018). One possibility is that POA$_{iso}$ neurons do not directly regulate USV production but

rather that reversible silencing of these neurons causes off-target disruptions of neural

activity in interconnected brain regions that in turn directly regulate USV production. Such a

relationship was demonstrated for motor cortex, whereby reversible silencing of motor cortex

disrupted performance of a learned forelimb reaching task in rats, while permanent lesions

of motor cortex had no effect on task performance after learning (Otchy et al., 2015).

However, our finding that optogenetic activation of POA$_{iso}$ neurons elicits USV production,

along with past work demonstrating that POA activation elicits USV production (Chen et al.,

2021; Gao et al., 2019; Karigo et al., 2021; Michael et al., 2020) supports the idea that the

POA directly regulates USV production. An alternative explanation for the apparently

contradictory effects of chemogenetic silencing vs. ablation of POA$_{iso}$ neurons is that while

the POA plays an obligatory role in mounting behavior (at least in certain contexts, see

below), compensatory changes in additional, non-POA circuits that promote USV production

(and social investigation) are sufficient to compensate for the permanent loss of POA$_{iso}$

neurons, leaving these behaviors unperturbed following POA$_{iso}$ neuronal ablation. The

identification of forebrain-to-midbrain circuits that regulate USV production in both females

and males remains an important future goal.

442   Our finding that POA$_{iso}$ neurons regulate female-female mounting extends recent

work examining the role of hypothalamic regions in regulating male mounting behavior in

different social contexts (Karigo et al., 2021). Although the POA plays a well-established role

in the regulation of mounting behavior by male rodents during interactions with females,

interactions which are typically marked by high rates of USV production and are considered

affiliative, the authors found that the VMH regulates male mounting behavior during

interactions with other males, which is typically not accompanied by USV production and

often precedes fighting. Taken together with the findings of the current study, we conclude

that the POA regulates mounting behavior in both male and female mice that is directed

toward female social partners and is accompanied by USV production. The question of

whether the social behaviors exhibited by single-housed female mice during same-sex

interactions are indeed affiliative and how these behaviors shape future interactions with

female social partners are important questions that remain to be addressed. Although

chemogenetic silencing of POA$_{iso}$ neurons tended to reduce female mounting and ablation of

POA$_{iso}$ neurons significantly reduced female mounting, we were not able to reliably elicit

mounting behavior through optogenetic activation of POA$_{iso}$ neurons (optogenetically-elicited

mounting was observed in n = 3 laser stimulations across N = 2 POA$_{iso}$-ChR2 females). We

note that previous studies that elicited mounting in male mice via optogenetic activation of

either Esr1^{+} POA neurons or POA neurons that co-express Esr1 and VGAT used longer

laser stimulation trains (15-30s) than we employed in the current study (Karigo et al., 2021;
Wei et al., 2018). It is possible that longer periods of optogenetic activation of POA$_{iso}$
neurons would be more effective in eliciting mounting in group-housed females, and another
possibility is that POA$_{iso}$ neurons overlap somewhat but not perfectly with the genetically-
defined groups of POA neurons manipulated in those prior studies. Regardless, our finding
that optogenetic activation of POA$_{iso}$ neurons promotes USV production efficaciously relative
to mounting indicates that effects on USV production are not secondary to effects on other
female social behaviors.

To understand whether a similar population of POA neurons might regulate changes
in social behavior following short-term isolation in males, we extended our experiments to
single-housed males that subsequently interacted with either a male social partner or with a
female social partner. Consistent with our prior work (Zhao et al., 2021), we found that short-
term isolation exerts more robust on the effects of male behavior during subsequent
interactions with females than during interactions with males. Although single-housed males
exhibited increased rates of social investigation while interacting with both males and
females relative to group-housed males, single-housed males that interacted with females
exhibited increases in all measured social behaviors (social investigation, USV production,
and mounting), a pattern of behavioral changes that is similar to what we observed in single-
housed females. We also observed that a population of POA neurons increased Fos
expression in single-housed males following interactions with female visitors but not
following interactions with male visitors. Unexpectedly, we found that the effects of
chemogenetically inhibiting POA$_{iso}$ neurons in males differed from those in females. Namely,
while reversible inhibition of POA$_{iso}$ neurons tended to reduce mounting during interactions
between single-housed subject males and female visitors, there were no effects on rates of
social investigation and USV production. These findings differ also from recent work showing
that chemogenetic inhibition (Chen et al., 2021) or optogenetic inhibition (Karigo et al., 2021)
of Esr1$^+$ POA neurons reduces USV production in male mice. Although the factors that
account for this difference in results are unclear, one possibility is that our TRAP2-based
viral labeling in males is biased toward POA neurons that regulate mounting as compared to
POA neurons that regulate USV production, although as stated above, more work is
required to understand whether these different social behaviors are regulated by distinct or
overlapping subsets of POA neurons. Future experiments can also explore to what extent
female and male POA$_{iso}$ neurons represent molecularly, anatomically, and functionally
similar or dissimilar neuronal populations.

The current study adds to an emerging body of literature implicating the POA in the

regulation of social behavior in both females and males, including in non-sexual social

contexts (Fukumitsu et al., 2022; Hu et al., 2021; Liu et al., 2023; McHenry et al., 2017; Wu

et al., 2021). Our findings also complement recent work that has described a role for the

POA in regulating behavioral responses to social isolation in female mice. A recent study

using BALB/c mice found that reunion with same-sex cagemates following short-term

"somatic isolation" (i.e., separation from cagemates via a partition that permits visual,

auditory, and olfactory signaling but prevents physical contact) increases the activity of

calcitonin-receptor (Calcr) expressing POA neurons (Fukumitsu et al., 2023). Knockdown of

Calcr expression in these neurons reduced social-seeking behaviors directed at the cage

partition exhibited by single-housed females, and chemogenetic activation of these neurons

increased partition-biting behavior in single-housed females. Another recent study using

female FVB/NJ mice applied a TRAP2-based intersectional approach to identify POA

neurons that increase their activity following reunion with same-sex cagemates ($MPN_{reunion}$

neurons) (Liu et al., 2023). Optogenetic activation of these neurons did not alter social

investigation in group-housed females, whereas activation of these neurons during reunion

with cagemates following short-term social isolation decreased social investigation.

However, optogenetic inhibition of $MPN_{reunion}$ during reunion did not alter rates of social

investigation in single-housed females. Although we did not test the effects of activating

POA_{iso} neurons in single-housed females in the current study, the difference in the effects of

inhibiting POA_{iso} neurons on the behavior of single-housed females (reduced rates of social

investigation, USV production, and mounting) relative to the effects of optogenetic inhibition

of $MPN_{reunion}$ neurons in Liu et al. (no effect on social investigation during reunion) suggest

that these may represent distinct populations of POA neurons. We note that in the current

study, single-housed female subjects remained single-housed for 24 hours following social

interaction TRAPing sessions, a design intended to maximize viral labeling of POA neurons

that promote increased female social behaviors and that would likely in turn minimize viral

labeling of POA neurons that promote social satiety. Taken together, these studies highlight

a complex role for the POA in regulating multiple aspects of changes in social behavior

following short-term social isolation. Although some studies of social isolation have avoided

the use of C57BL/6J female mice, the robust triad of changes in social behavior exhibited by

these females following short-term isolation affords a powerful opportunity to continue

investigating neural circuit mechanisms through which short-term social isolation promotes

social behaviors, as well as to investigate how hypothalamic circuits regulate the

coordinated production of suites of social behaviors during female-female social interactions.

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the experiments. X.Z., Y.C., D.S., V.C., D.D., J.W.S., A.S., and K.A.T. analyzed data. X.Z.

and K.A.T. wrote the manuscript, and all authors approved the final version.

Declaration of Interests: The authors declare no competing interests.

Figure legends

Figure 1. The POA contains neurons that increase Fos expression in single-housed females following same-sex interactions. (A) Schematic of experiment to measure Fos expression in group-housed and single-housed females following same-sex social interactions. (B) Total time spent engaged in resident-initiated social investigation for group-housed residents (teal) and single-housed residents (maroon). (C) Same as (B), for total resident-initiated mounting time. (D) Same as (B), for total USVs recorded from pairs containing group-housed or single-housed residents. (E) Left-most image shows the location of the POA in a coronal brain section. Representative confocal images show Fos expression (green) in the POA of a group-housed female (left) and a single-housed female (right) following same-sex social interactions. Blue, Neurotrace. (F) Quantification of Fos-positive neurons is shown for the POA (left), the VMH (middle), and the caudolateral PAG (right) for group-housed and single-housed females. Open bars show data from females that did not engage in social interactions with novel females (baseline), and closed bars show data from females following social interactions with novel females (interaction). (G) Total time spent in resident-initiated interaction is plotted for 14-day single-housed (maroon) and re-group-housed females (teal) during same-sex interactions that occurred prior to isolation (day 0), following 3 days of isolation (day 3), and on the test day (day 17). (H) Same as (G), for total resident-initiated mounting time. (I) Same as (G), for total USVs. (J) Quantification of Fos-positive POA neurons is shown for 14-day single-housed females (maroon) and re-group-housed females (teal).

Figure 2. Effects of chemogenetic inhibition of POA_{iso} neurons on the social behaviors of single-housed female mice. (A) Experimental timeline and viral strategy to chemogenetically inhibit the activity of POA_{iso} neurons in single-housed females. (B) Total time spent in resident-initiated social investigation is shown on saline and CNO days for 4 experimental groups: (red symbols) experimental females in which hM4Di is expressed in POA_{iso} neurons; (black symbols) control females in which GFP is expressed in POA_{iso} neurons; (brown symbols) control females in which hM4Di is expressed in 'TRAPed' AH neurons; (gray symbols) control females in which hM4Di is expressed in 'TRAPed' VMH neurons. (C) Same as (B), for total duration of resident-initiated mounting. (D) Same as (B), for total USVs. (E) Total movement is plotted for females with hM4Di expressed in POA_{iso} neurons, on saline days vs. CNO days.

Figure 3. Effects of caspase-mediated ablation of POA_{iso} neurons on the social behaviors of single-housed female mice. (A) Experimental timeline and viral strategy for caspase-mediated ablation of POA_{iso} neurons in single-housed females. (B) Total time spent

in resident-initiated social investigation is shown pre- and post-4-OHT treatment for 2

experimental groups: (red symbols) experimental females in which caspase is expressed in

POA$_{iso}$ neurons; (black symbols) control females in which GFP is expressed in POA$_{iso}$

neurons. (C) Same as (B), for total duration of resident-initiated mounting. (D) Same as (B),

for total USVs.

Figure 4. Effects of optogenetic activation of POA$_{iso}$ neurons on the behaviors of

group-housed female mice. (A) Experimental timeline and viral strategy to optogenetically

activate POA$_{iso}$ neurons in group-housed females. (B) Mean total USVs produced during

social sessions shown for pre-laser periods and during laser stimulation periods for

experimental females with ChR2 expressed in POA$_{iso}$ neurons (red symbols; N = 9) and for

control females with GFP expressed in POA$_{iso}$ neurons (black symbols, N = 6). (C) Same as

(B), for social sessions. (D) Spectrograms are shown from a representative POA$_{iso}$-ChR2

female to illustrate USVs that were elicited through optogenetic activation of POA$_{iso}$ neurons

in a solo session (top) and a social session (bottom). Blue bars indicate timing of laser

stimulation.

Figure 5. Context-dependent differences in POA Fos expression and effects of

chemogenetic inhibition of POA$_{iso}$ neurons on the social behaviors of single-housed

male mice. (A) Total time spent in resident-initiated social investigation is shown for group-

housed male residents (teal) and single-housed male residents (maroon) during interactions

with either female visitors (left) or male visitors (right). (B) Same as (A), for total resident-

initiated mounting time. (C) Same as (A), for total USVs recorded from pairs containing

group-housed or single-housed male residents. (D) Total number of Fos-positive POA

neurons is shown for group-housed male residents (teal) and single-housed male residents

(maroon) following interactions with female visitors (left) or male visitors (right). (E)

Experimental timeline and viral strategy to chemogenetically inhibit the activity of POA$_{iso}$

neurons in single-housed males. (F) Total time spent in resident-initiated social investigation

is shown on saline and CNO days for experimental males in which hM4Di is expressed in

POA$_{iso}$ neurons (red symbols, N = 7) and control males in which GFP is expressed in POA$_{iso}$

neurons (black symbols, N = 7). (G) Same as (F), for total duration of resident-initiated

mounting. (H) Same as (F), for total USVs.

Materials and methods

Key Resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
c-Fos (9F6) Rabbit mAb	Cell Signaling Technology	2250S
Alexa Fluor 488 goat-anti-rabbit	Invitrogen	A-11008
Neurotrace 435/455 Blue	Thermo Fischer Scientific	CAT#: N21479
Commercial assay or kit		
HCR v3.0	Molecular Instruments	
Bacterial and Virus Strains		
AAV2/1-hSyn-FLEX-hM4Di-mCherry	Addgene	RRID: Addgene_44262
AAV2/1-Ef1alpha-hChR2(h134R)-EYFP-WPRE-HGHpA	Addgene	RRID: Addgene_20298
AAVrg-pgk-Cre	Addgene	RRID: Addgene_24593
pAAV-flex-taCasp3-TEVp	Addgene	RRID: Addgene_45580
AAV2/1-pCAG-FLEX-EGFP-WPRE	Addgene	RRID: Addgene_51502
Chemicals, Peptides, and Recombinant Proteins		
Clozapine N-oxide dihydrochloride	Hello Bio	HB6149; E1458-2-1
4-Hydroxytamoxifen	Hello Bio	HB6040
Experimental Models: Organisms/Strains		
TRAP2 Fos$^{tm2.1(icre/ERT2)Luo}$/J	Jackson Labs	IMSR_JAX:030323
Ai14 B6.Cg$Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze}$/J	Jackson Labs	IMSR_JAX:007914
C57BL/6J	Jackson Labs	IMSR_JAX:000664
Software and Algorithms		
MATLAB	Mathworks	http://www.mathworks.com RRID: SCR_001622
ZEN	Zeiss	https://www.zeiss.com RRID:SCR_013672
Spike2	CED	http://ced.co.uk RRID:SCR_000903
ImageJ	NIH	https://imagej.net/ij/ RRID:SCR_003070
Behavioral Observation Research Interactive Software	Open Behavior	https://github.com/olivierfriard/BORIS RRID:SCR_021509
R Project for Statistical Computing	R Core Team	http://www.r-project.org/ RRID:SCR_001905
R Studio	Posit	https://posit.co/ RRID:SCR_000432

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by

the lead contact, Katherine Tschida (kat227@cornell.edu).

Experimental models and subject details

Animal statement

All experiments and procedures were conducted according to protocols approved by the
Cornell University Institutional Animal Care and Use Committee (protocol #2020-001).

Animals

TRAP2 (Jackson Laboratories, 030323) and Ai14 (Jackson Laboratories, 007914) mice were
at least 8 weeks old at the time of the experiments or surgeries. TRAP2;Ai14 mice were
generated by crossing TRAP2 with Ai14. All mice were kept on a 12:12 reversed light/dark
cycle, were housed in ventilated micro-isolator cages in a controlled environment with
regulated temperature and humidity, and were provided with unrestricted access to food and
water. A running wheel (Innovive) was present in all homecages from the time of weaning
and was subsequently removed immediately before initiating the social interaction test.
Mouse cages were cleaned weekly, and experiments were never conducted on cage change
days.

Methods details

Social isolation and social interaction tests

Female and male subject mice were either group-housed with same-sex siblings or
separated from their cage mates and individually housed in clean cages for three days prior
to behavioral tests. In the case of group-housed subject mice, siblings were temporarily
removed from the home cage for the duration of the test. The subject animal's home cage
was then placed in a sound-attenuating recording chamber (Med Associates) equipped with
an ultrasonic microphone (Avisoft), an infrared light source (Tendelux), and a webcam
(Logitech, with the infrared filter removed to enable video recording under infrared lighting
conditions). A novel, group-housed visitor mouse (female or male mouse on a C57BL/6
background) was placed in the home cage of the subject mouse, and vocal and non-vocal
behaviors were recorded for 30 minutes. Visitor mice were used across multiple experiments
(< 6 in total), including in interactions with both group-housed and single-housed subject
mice. Visitor females used in male-female interactions were never used for female-female
experiments, but a subset of visitors used in female-female interactions were subsequently
used in male-female experiments.

A separate cohort of female mice was used to investigate the effects on social behavior of
re-group-housing following a period of social isolation. For this cohort of mice, social

interaction tests with novel, group-housed female visitors were conducted at three
timepoints: (1) on day 0, when subject females were still group-housed; (2) On day 3, after
being single-housed for 3 days; (3) on day 17, after a randomly selected subset of subject
females were re-group-housed with their siblings for two weeks, and the remaining female
subjects remained single-housed for two weeks.

USV recording and detection

USVs were recorded with an ultrasonic microphone (Avisoft, CMPA/CM16), amplified
(Presonus TubePreV2), and digitized at 250 kHz (Avisoft UltrasoundGate 166H or CED
Power 1401). USVs were detected with custom MATLAB codes (Tschida et al., 2019) using
the following parameters (mean frequency > 45 kHz; spectral purity > 0.3; spectral
discontinuity < 1.00; minimum USV duration = 5 ms; minimum inter-syllable interval = 30
ms).

Analyses of non-vocal social behaviors

Trained observers used BORIS software (v.8.13; Friard and Gamba, 2016) to score the
following non-vocal behaviors: resident-initiated social investigation and resident-initiated
mounting. Social investigation included sniffing and following. Resident-initiated mounting of
the visitor typically occurred following a period of resident-initiated social investigation, with
the resident mouse positioning its forelimbs on top of the body of the visitor, sometimes with
pelvic thrusts and sometimes without. Neither visitor-initiated mounting nor fighting were
observed in our dataset.

In some trials, total movement was estimated using a custom MATLAB code that allows the
user to mark the position of a mouse in every 30th frame (i.e., once per second). Total
movement was then calculated as the sum of changes in position across pairs of marked
frames.

Fos immunohistochemistry

Two hours following the start of the social interaction test, mice were deeply anesthetized
using isoflurane and then transcardially perfused with phosphate-buffered saline (PBS, pH
7.4), followed by 4% paraformaldehyde (PFA; Sigma-Aldrich, in 0.1 M PBS, pH 7.4). Brains
were subsequently dissected and post-fixed in 4% PFA for 24 hours at 4°C, followed by
immersion in 30% sucrose solution in PBS for 48 hours at 4°C. Afterward, brains were
embedded in frozen section embedding medium (Surgipath, VWR), flash frozen in a dry ice-
ethanol (100%) bath, and then stored at –80°C until sectioning. Sections were cut on a
cryostat (Leica CM1950) to a thickness of 80 μm, washed in PBS (3 x 5 mins at RT),

permeabilized for 2-3 hours in PBS containing 1% Triton X-100 (PBST), and then blocked in

0.3% PBST containing 10% Blocking One (Nacalai USA) for 1 hour at RT on a shaker.

Sections were then incubated for 24 hours at 4°C with primary antibody in blocking solution

(1:1000 rabbit-anti-Fos, Cell Signaling Technologies, 2250S), washed 3 x 30 minutes in

0.3% PBST, then incubated for 24 hours at 4°C with secondary antibody in blocking solution

(1:1000, Alexa Fluor 488 goat-anti-rabbit, Invitrogen, plus 1:500 NeuroTrace, Invitrogen)

Finally, sections were washed for 2 x 10 minutes in 0.3% PBST, followed by washing for 2 x

10 minutes in PBS. After mounting on slides, sections were dried and coverslipped with

Fluromount G (Southern Biotech). Slides were imaged with a 10x objective on a Zeiss 900

laser scanning confocal microscope, and Fos-positive neurons within regions of interest

were counted manually by trained observers.

Floating section two-color in situ hybridization

In situ hybridization was conducted using hybridization chain reaction (HCR v3.0, Molecular

Instruments). Ten minutes after the completion of the 30-minute social interaction tests, mice

underwent transcardial perfusion with RNase-free PBS (DEPC-treated), followed by 4%

PFA. Dissected brain samples were post-fixed overnight in 4% PFA at 4°C, cryoprotected in

a 30% sucrose solution in DEPC-PBS at 4°C for 48 hours, flash frozen in section embedding

medium, and stored at –80°C until sectioning. 40 µm-thick coronal floating sections were

collected into sterile 24-well plates in DEPC-PBS. These sections were briefly fixed once

again for 5 minutes in 4% PFA and subsequently immersed in 70% EtOH in DEPC-PBS

overnight. Sections were then rinsed in DEPC-PBS, incubated for 45 minutes in 5% SDS in

DEPC-PBS, followed by a series of rinses and incubations: 2x SSCT, pre-incubation in HCR

hybridization buffer at 37°C, and incubation in HCR hybridization buffer containing RNA

probes (VGAT and Fos) overnight at 37°C. Sections were then rinsed 4 x 15 minutes at

37°C in HCR probe wash buffer, rinsed in 2X SSCT, pre-incubated in HCR amplification

buffer, and then incubated in HCR amplification buffer containing HCR amplifiers at RT for

approximately 48 hours. On the final day, sections were rinsed in 2x SSCT, counterstained

with DAPI (Thermo Fisher, 1:5000), rinsed again with 2x SSCT, mounted on slides, and

coverslipped with Fluoromount-G (Southern Biotech). After drying, slides were imaged with a

10x or 20x objective on a Zeiss 900 laser scanning microscope. Neurons were scored from

three sections of tissue from the POA from each mouse, and the absence of presence of

staining for different probes was quantified manually by trained scorers.

Viruses

The following viruses and injection volumes were used: AAV2/1-hSyn-FLEX-hM4Di-mCherry

(Addgene #44262, 200 nL), AAV2/1-CAG-FLEX-EGFP-WPRE (Addgene #51502, 200 nL),

AAV2/5-Ef1alpha-FLEX-taCasp3-TEVp (Addgene #45580, 200 nL), AAV2/1-Ef1alpha-

hChR2(h134R)-EYFP-WPRE-HGHpA (Addgene #20298, 200 nL), AAVrg-pgk-Cre (Addgene

#24593, 200 nL). The final injection coordinates were as follows: POA, AP = -0.1 mm, ML =

0.6 mm, DV = 5.1 mm; AH, AP = -0.7 mm, ML = 0.6 mm, DV = 5.1 mm; VMH, AP = -1.5 mm,

ML = 0.7 mm, DV = 5.4 mm; PAG, AP = -4.7 mm, ML = 0.6 mm, DV = 1.75 mm. Viruses

were pressure-injected using a pulled glass pipettes mounted in a programmable nanoliter

injector (Nanoject III, Drummond) at a rate of 15 nL every 60 s.

Stereotaxic Surgery

Mice were anesthetized using isoflurane (2.5% for induction, then 1.5 - 2.5% for

maintenance) and then securely positioned in a stereotaxic apparatus (Angle Two, Leica). A

midline incision in the scalp was made to expose the skull, and small craniotomies were

created dorsal to each injection site using a surgical drill. Viral injection pipettes were left in

place for a minimum of 10 minutes before and after viral injections to minimize backflow

upon pipette withdrawal from the brain. Surgical sutures (LOOK 774B, Fisher Scientific) and

tissue adhesive (3M) were used to close the incision.

For optogenetic activation experiments, an optogenetic ferrule (RWD Fiber Optic Cannula,

Ø1.25 mm Ceramic Ferrule, 200 μm Core, 0.22 NA, L = 7 mm) was implanted approximately

250 μm above the viral injection site immediately following the viral injection and was

secured in place with Metabond (Parkell).

TRAP activity-dependent labeling

Solutions of 4-hydroxytamoxifen (4-OHT, HelloBio, HB6040) were prepared by dissolving 4-

OHT powder at 20 mg/mL in ethanol by shaking at 37°C, and aliquots (75 uL) were then

stored at -20°C. Before use, 4-OHT was redissolved in ethanol by shaking at 37°C and

filtered corn oil was added (150 uL). Ethanol was then evaporated by vacuum under

centrifugation to give a final concentration of 10 mg/mL, and the 4-OHT solution was used

on the same day it was prepared.

To express viral transgenes in recently active neurons, we used the Targeted

Recombination in Active Populations (TRAP2) strategy. Two weeks following viral injection,

TRAP2 and TRAP2;Ai14 mice were given 30-minute social encounters (as described

above). Following the social encounter, subject mice received I.P. injections of 4-OHT (50

mg/kg) to enable expression of viral transgenes in recently active neurons. To minimize

neural activity triggered by stimuli outside of the social interaction test, all subject animals

were individually housed for an additional 24 hours following 4-OHT treatment before being

re-group-housed with their same-sex siblings.

Chemogenetic inhibition

To reversibly reduce neuronal activity, TRAP2 female mice received bilateral injections of an

Cre-dependent inhibitory DREADDs virus into the hypothalamus (AAV2/1-hSyn-FLEX-

hM4Di-mCherry; injected into the POA, AH, or VMH) as described above. TRAP2 male mice

received the same viral injections into the POA only. Three weeks later, mice were single-

housed for 3 days and then were subsequently given a 30-minute social encounter with a

novel, group-housed female visitor. Subject mice then received an I.P. injection of 4-OHT to

enable expression of hM4Di in activity-defined populations of hypothalamic neurons. Two

weeks later, subject mice received an I.P. injection of either sterile saline (as a control) or

clozapine-n-oxide (CNO, 4 mg/kg, Hello Bio HB6149; to inhibit neurons expressing hM4Di)

30 minutes prior to a social interaction test. Three days later, mice that previously were

treated with saline received an I.P. injection of CNO, and mice that were previously treated

with CNO received an I.P. injection of saline, 30 minutes prior to another social interaction.

Rates of USV production and non-vocal social behaviors were compared between saline

and CNO days within animals to assess the effects of neuronal inhibition on social

behaviors. Control mice received unilateral injections into the POA of a Cre-dependent AAV

driving the expression of GFP (AAV2/1-CAG-FLEX-EGFP) and were otherwise treated

identically to experimental animals.

Neuronal ablation

To permanently ablate neurons, TRAP2;Ai14 female mice received bilateral injections of an

AAV2/5-ef1alpha-FLEX-taCasp3-TEVp virus into the POA. Following a three-week recovery

period, these animals were individually housed for three days and subsequently given a 30-

minute social encounter with a novel, group-housed female visitor. Subject mice then

received an I.P. injection of 4-OHT to enable expression of caspase in activity-defined POA

neurons. Two weeks later, females were single-housed for three days and then given a

second 30-minute social interaction test. Social behaviors of subject females were compared

between the pre-ablation and post-ablation interaction tests to assess the effects of neuronal

ablation.

Optogenetic activation

Female TRAP2 mice received unilateral injections into the POA of AAV-ef1α-FLEX-ChR2

(experimental) or AAV-CAG-FLEX-GFP (control). In the same surgery, an optogenetic

ferrule was implanted approximately 250 μm above the viral injection site. Three weeks later,

females were single-housed for 3 days and then given a 30-minute social interaction with a

novel group-housed, female visitor. Subject females then received an I.P. injection of 4-

OHT. Two weeks later, females were first placed alone in the testing chamber for a 5-minute

habituation period after connecting the laser patch cable to the female's optogenetic ferrule.

Optogenetic activation sessions consisted of a 5-minute period in which optogenetic

activation was performed in solo females, followed by a 20-minute period in which activation

was performed as subject females interacted with a novel, group-housed female visitor. The

social session was further divided into three phases: 5 minutes without optogenetic

activation, 10 minutes with optogenetic activation, and 5 minutes without optogenetic

activation. During the middle 10 minutes of the social session, some laser stimuli were

delivered at times when the two females were near to one another (inter-animal distance <

~2 mouse body lengths), and other stimuli were delivered at times when the females were

not in close contact. POA_{iso} neurons were optogenetically activated with illumination from a

473 nM laser (10 mW) at 20-50 Hz (10-20 ms pulses, trains lasted 5-10s) Laser stimuli were

driven by computer-controlled voltage pulses (Spike 2 version 10.8, CED).

Anatomical tracing

Female TRAP2 mice used as GFP controls in the chemogenetic inhibition experiments were

subsequently used for anterograde mapping of the axonal projections of POA_{iso} neurons.

Three weeks following unilateral injection into the POA of a Cre-dependent AAV driving the

expression of GFP (AAV2/1-CAG-FLEX-EGFP), females were single-housed for 3 days and

subsequently given a 30-minute social interaction test. Subject mice then received an I.P.

injection of 4-OHT. Six weeks later, females were perfused, brains were collected and

sectioned, and a confocal microscope was used to image GFP-positive axon terminals

within coronal brain tissue sections.

To examine the overlap between PAG-projecting POA neurons and Fos expression, Ai14

females first received a unilateral injection into the PAG of an AAV driving the retrograde

expression of Cre-recombinase (AAVrg-pgk-Cre). Two weeks later, these females were

given a 30-minute social interaction test. Ninety minutes after the test, subject females were

perfused, brains were collected, and coronal brain sections containing the POA were

collected for Fos immunohistochemistry as described above. Brain tissue sections were

imaged with a 10x objective on a Zeiss 900 laser scanning confocal microscope, and POA

neurons that were Fos-positive and tdTomato-positive were counted manually by trained

observers.

Statistics

Two-sided statistical comparisons were used in all analyses (alpha = 0.05). The Shapiro-

Wilk test was performed to analyze the normality of each data distribution, and non-

parametric statistical tests were used for comparisons that included non-normally distributed

data. No statistical methods were used to pre-determine sample size. Mice were only

excluded from analysis in cases in which viral injections were not targeted accurately.

Details of the statistical analyses used in this study are included in Table S1.

Supplemental Information

Figure S1. Additional characterization of Fos expression in single-housed vs. group-
housed females and comparison to rates of female social behaviors. (A)
Representative confocal images show Fos expression (green) in the VMH of a group-
housed female (left) and a single-housed female (right) following same-sex social
interactions. Blue, Neurotrace. (B) Same as (A), for the caudolateral PAG. (C) Left, the
relationship between total time spent in resident-initiated social investigation and numbers of
Fos-positive POA neurons is shown for group-housed (teal) and single-housed (maroon)
female residents following interactions with novel females. Middle, same as left, for total
resident-initiated mounting time vs. numbers of Fos-positive POA neurons. Data only shown
for single-housed residents because group-housed residents never mounted female visitors.
Right, same as left, for total USVs vs. numbers of Fos-positive POA neurons. (D) Left, the
relationship between total time spent in resident-initiated social investigation and numbers of
Fos-positive VMH neurons is shown for group-housed (teal) and single-housed (maroon)
female residents following interactions with novel females. Middle, same as left, for total
resident-initiated mounting time vs. numbers of Fos-positive VMH neurons. Right, same as
left, for total USVs vs. numbers of Fos-positive VMH neurons. (E) Left, the relationship
between total time spent in resident-initiated social investigation and numbers of Fos-
positive caudolateral PAG neurons is shown for group-housed (teal) and single-housed
(maroon) female residents following interactions with novel females. Middle, same as left, for
total resident-initiated mounting time vs. numbers of Fos-positive PAG neurons. Right, same
as left, for total USVs vs. numbers of Fos-positive PAG neurons.

Figure S2. Characterization of neurotransmitter phenotype and axonal projections of
POA_{iso} neurons. (A) Representative confocal images of in situ hybridization performed on
brain sections containing the POA, showing overlap of expression of Fos (green) and VGAT
(magenta). Blue, DAPI. (B) Quantification of proportion of Fos-positive POA neurons that
expressed VGAT. (C) Experimental timeline and viral strategy to express GFP in POA_{iso}
neurons. (D) Confocal images showing GFP-labeled axons of POA_{iso} neurons within the
caudolateral PAG. Blue, Neurotrace. (E) Experimental timeline and viral strategy to
retrogradely label PAG-projecting POA neurons with tdTomato. (F) Confocal image showing
tdTomato labeling in a coronal section containing the POA, and dotted circles in insets
indicate examples of double-labeled neurons. Neurotrace, blue. (G) Quantification of
proportion of tdTomato-expressing POA neurons that are also Fos-positive.

Table S1. Details of statistical analyses.

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Figure 1.

Figure 2.

Figure 3.

A

AAV2/5-Ef1alpha-FLEX-taCasp3

3 wk recovery

3d Iso

Pre-ablation Test (30 min)

4-OHT

2 wk Re-group -housed

3d Iso

Post-ablation Test (30 min)

B

Resident-initiated investigation (s)

Post > Pre, p < 0.05

Pre Post | Pre Post

POA_iso caspase   POA_iso GFP

C

Total mounting duration

13/15 | 4/15 | 8/13 | 6/13

Pre Post | Pre Post

n.s.

POA_iso caspase   POA_iso GFP

D

Total USVs

n.s. | n.s.

Pre Post | Pre Post

POA_iso caspase   POA_iso GFP

Figure 4

A

AAV2/1-Ef1alpha-hChR2

3 wk recovery → 3d Iso → 30-min ♀-♀ interaction → 4-OHT → 2 wk group housed → Solo (5 min Laser on) → Social (5 min Laser off | 10 min On | 5 min Off)

B Solo session

Pre Post Pre Post

n.s.

n.s.

USVs per second laser stimulation

POA$_{iso}$ ChR2   POA$_{iso}$ GFP

C Social session

Pre Post Pre Post

**

n.s.

USVs per second laser stimulation

POA$_{iso}$ ChR2   POA$_{iso}$ GFP

D Laser on

Solo   120 kHz ... 0   1 s

Social   120 kHz ... 0   1 s

Figure 5.