

Serotonin signaling in the brain of adult female mice is required for sexual preference

Shasha Zhang^{a,b,1}, Yan Liu^{a,b,1}, and Yi Rao^{a,b,2}

^aState Key Laboratory of Biomembrane and Membrane Biotechnology and Peking-Tsinghua Center for Life Sciences, School of Life Sciences, and Peking University-International Data Group-McGovern Institute for Brain Research, Peking University, Beijing 100871, China; and ^bNational Institute of Biological Sciences, Beijing 102206, China

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A role for serotonin in male sexual preference was recently uncovered by our finding that male mutant mice lacking serotonin have lost sexual preference. Here we show that female mouse mutants lacking either central serotonergic neurons or serotonin prefer female over male genital odors when given a choice, and displayed increased female–female mounting when presented either with a choice of a male and a female target or only with a female target. Pharmacological manipulations and genetic rescue experiments showed that serotonin is required in adults. Behavioral changes caused by deficient serotonergic signaling were not due to changes in plasma concentrations of sex hormones. We demonstrate that a genetic manipulation reverses sexual preference without involving sex hormones. Our results indicate that serotonin controls sexual preference.

sexual behaviors | neurotransmitter | Tph2 knockout

Sexual behaviors are among the most important social behaviors. Although preference for the opposite sex is essential for reproduction, sexual behaviors toward members of the same sex have been observed in many animal species, indicating that there are potential evolutionary advantages (1, 2). The diversity of sexual preference has been of scientific interest to scholars from Aristotle to present-day scientists (2, 3).

Biologically, changes in sex hormones can change sexual behavior or sexual preference, resulting in either a loss of sexual preference or a reversal of sexual preference (4–14). Although a genetic component for homosexual orientation has been suggested (15), no specific genes have been identified in sexual preference in humans (16–20).

Same-sex preference was reported in female mice lacking the gene encoding estrogen-binding plasma protein alpha-fetoprotein (AFP) or those lacking the gene for aromatase through indirect effects on sex hormones (11, 12, 14, 21). Pheromone perception is important for sexual behaviors. Surgical removal of the vomeronasal organ or genetic inactivation of transient receptor potential channel 2 (*Tprc2*), which encodes a cation channel in the vomeronasal organ (22, 23), or cyclic nucleotide-gated channel $\alpha 2$ (*Cgna2*) in the main olfactory epithelium (24) resulted in loss of sexual preference in male mice. *Tprc2*^{-/-} mutant females showed female–female mounting behavior (13, 25). There was an overall reduction of sexual behavior in *Cgna2* mutant mice (19). However, none of these mice has been shown to prefer the same sex.

Our recent genetic studies have shown that 5-hydroxytryptamine (5-HT) in the male mouse brain is required for sexual preference because there was no sexual preference in mutant male mice lacking serotonergic neurons or 5-HT (26). We have now carried out experiments to determine whether 5-HT is involved in female sexual preference. Here we report a crucial role of serotonergic signaling in female sexual preference: strikingly, sexually differential olfactory preference indicated by several assays was reversed in female mice lacking serotonergic neurons or those unable to synthesize 5-HT in the brain. Our results suggest a role for 5-HT in sexual preference is separate from roles in sexual drive and discrimination. These studies have furthered our understanding of molecular mechanisms underlying neural control of sexual preference.

Results

Female Mice Lacking Serotonergic Neurons Preferred Female Over Male Mice. It was known that conditional LIM homeobox transcription factor 1-beta (*Lmx1b*) knockout mice (*Lmx1b*^{-/-}) could be generated by crossing Cre recombinase driven by Pet-1 enhancer (*ePet-Cre*) into *Lmx1b*^{flloxP/flloxP} mice, leading to the absence of serotonergic neurons in the brain without affecting 5-HT in the periphery (27). We have obtained female *Lmx1b*^{-/-} mice and confirmed that the levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were lower in the brains of homozygous (*Lmx1b*^{-/-}) mutant females than those in the WT (*Lmx1b*^{+/+}) and heterozygous (*Lmx1b*^{+/-}) females (Fig. S1A and B). The level of 5-HT in heterozygous females was also lower than that in the WT.

Sexual preference was first investigated by presenting a male and a female target mouse to a test female mouse. We have observed that head and genital areas in male rodents are highly attractive to WT female rodents, which is likely due to the presence of pheromones in exocrine glands in those areas (28, 29). We measured the latency, frequency, and duration of females attempting to sniff the genital and head areas of male and female targets. It was clear that WT females preferred male head and genital areas over female head and genital areas, whereas *Lmx1b*^{-/-} females showed the opposite preference. The latency for sniffing target mice was not different among the *Lmx1b*^{+/+}, *Lmx1b*^{+/-}, and *Lmx1b*^{-/-} females (Fig. S2A). When the duration of sniffing the whole body was compared, *Lmx1b*^{+/+} and *Lmx1b*^{+/-} females spent longer time sniffing male target mice than female targets (Fig. 1A and Fig. S2C). By contrast, *Lmx1b*^{-/-} females sniffed female targets longer than male targets (Fig. 1A and Fig. S2C). In sniff bouts, *Lmx1b*^{+/+} and *Lmx1b*^{+/-} females showed preference for males over females, whereas *Lmx1b*^{-/-} females showed preference for females over males (Fig. S2B).

The reversal of sexual preference was particularly obvious when sniffing of the genital and head areas was analyzed separately from the rest of the body. *Lmx1b*^{-/-} mice showed a shorter latency to approach and sniff the genital area of female targets than that of male targets, whereas *Lmx1b*^{+/+} or *Lmx1b*^{+/-} females did not (Fig. S2D). *Lmx1b*^{+/+} female littermates showed significant preference for male over female genital areas, both in the number of sniffing bouts (Fig. 1B and Fig. S2E) and in sniffing duration (Fig. 1C and Fig. S2F). This preference was reversed in *Lmx1b*^{-/-} females (Fig. 1B and C and Fig. S2E and F): they sniffed the female genital area more frequently and with longer duration than the male genital area. Compared with their *Lmx1b*^{+/+} and *Lmx1b*^{+/-} littermates, a significantly smaller percentage of *Lmx1b*^{-/-} females preferred the male over female

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¹S.Z. and Y.L. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: yrao@pku.edu.cn.

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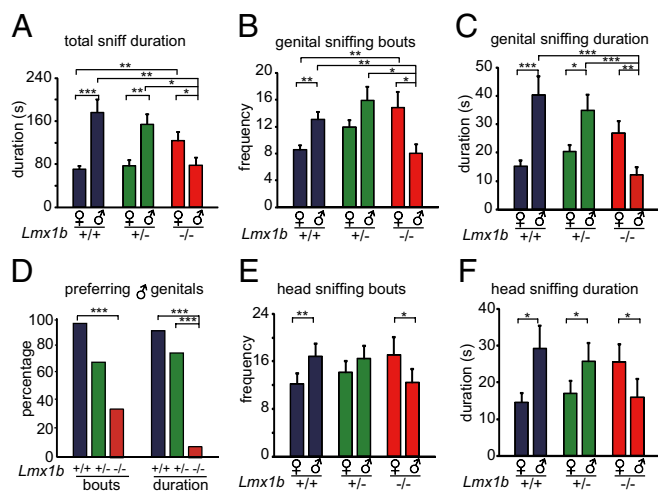


Fig. 1. *Lmx1b*^{-/-} female mice preferred female over male mice. A test female was presented with a male and a female target. $n = 18$ for *Lmx1b*^{+/+} (^{+/+}), $n = 15$ for *Lmx1b*^{+/-} (^{+/-}), $n = 15$ for *Lmx1b*^{-/-} (^{-/-}). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (A) *Lmx1b*^{-/-} females sniffed females longer than males, whereas their *Lmx1b*^{+/+} and *Lmx1b*^{+/-} female littermates sniffed males longer. *Lmx1b*^{-/-} females sniffed males for a shorter time than their female littermates. *Lmx1b*^{-/-} female mice sniffed females longer than their *Lmx1b*^{+/+} littermates. (B) *Lmx1b*^{-/-} females sniffed female genitals more often than male genitals, whereas their *Lmx1b*^{+/+} and *Lmx1b*^{+/-} female littermates sniffed male genitals more often than female genitals. *Lmx1b*^{-/-} female littermates did not show sexual preference in sniff bouts. *Lmx1b*^{-/-} females sniffed male genitals less and female genitals more than *Lmx1b*^{+/+} females. (C) *Lmx1b*^{-/-} females sniffed female genitals longer than male genitals, whereas their *Lmx1b*^{+/+} and *Lmx1b*^{+/-} female littermates sniffed male genitals longer than female genitals. There is no significant difference among *Lmx1b*^{-/-}, *Lmx1b*^{+/+}, and *Lmx1b*^{+/-} females in the duration of sniffing female genitals, but *Lmx1b*^{-/-} females sniffed male genitals for a shorter time than their female littermates. (D) Percentage of mice of each genotype that sniffed male genitals more (bouts) or longer (duration) than female genitals. (E) *Lmx1b*^{-/-} females sniffed female heads more than male heads, whereas their *Lmx1b*^{+/+} female littermates sniffed male heads more. *Lmx1b*^{+/-} females had an intermediate phenotype: they had no preference for either males or females. (F) *Lmx1b*^{-/-} females sniffed female heads for a longer duration than male heads, whereas their *Lmx1b*^{+/+} and *Lmx1b*^{+/-} female littermates sniffed male heads longer than female heads.

genital area (Fig. 1D). *Lmx1b*^{-/-} female mice also showed reversed preference of head sniffing; *Lmx1b*^{-/-} sniffed female heads more frequently (Fig. 1E and Fig. S2G) and for longer duration than male heads (Fig. 1F and Fig. S2H).

An intermediate phenotype was detected in *Lmx1b*^{+/-} females: they were similar to *Lmx1b*^{+/+} females in sniff duration preference (Fig. 1A, C, and F and Fig. S2F), but their preference was not statistically significant when the preference was analyzed with sniff bout frequency (Fig. 1B and E). This is consistent with an intermediate level of 5-HT in *Lmx1b*^{+/-} females (Fig. S1A), suggesting that this phenotype is sensitive to the dosage of 5-HT. The dosage sensitivity was also observed in some of the other assays (Fig. 2B).

These results indicate that mutant females lacking serotonergic neurons showed preference for females over males.

Preference of Female Mice Lacking Serotonergic Neurons for Female vs. Male Genital Odors. When presented with live animals, the selection of sexual partners by rodents will be affected by behavioral feedback from target mice and by olfactory, visual, and acoustic cues of targets (1, 30). To avoid behavioral feedback from live animals and to examine pheromonal preference specifically, we used the genital odor assay to study the preference of female mice for male and female genital excretions.

As known previously (31), WT females were more attracted by pheromones present in the genital areas of the opposite sex than by those from the same sex: when given a choice between adult male genital excretion and estrous female genital excretion smeared on two sides of a slide, more *Lmx1b*^{+/+} and *Lmx1b*^{+/-} females sniffed genital odor from males longer than from that of estrous females (Fig. 2A), whereas a significantly lower percentage of *Lmx1b*^{-/-} females sniffed male genital odor longer than female genital odor (Fig. 2A). *Lmx1b*^{+/+} females sniffed male genital odor longer than female genital odor (Fig. 2B). *Lmx1b*^{+/-} females sniffed male and female genital odor equivalently (Fig. 2B). *Lmx1b*^{-/-} females sniffed female genital odor longer than male genital odor (Fig. 2B). *Lmx1b*^{-/-} females sniffed male genital odor for a shorter duration than did the WT female littermates (Fig. 2B). By examining the difference in sniff duration of individual females, we also observed that *Lmx1b*^{-/-} females were significantly different from *Lmx1b*^{+/+} and *Lmx1b*^{+/-} females (Fig. 2C). The sniff latency of *Lmx1b*^{-/-} mice was not significantly different from those of *Lmx1b*^{+/+} and *Lmx1b*^{+/-} female littermates (Fig. S3A). When choosing between genital odors from diestrous females and males, *Lmx1b*^{-/-} females also showed same-sex preference, whereas *Lmx1b*^{+/+} and *Lmx1b*^{+/-} females showed no preference (Fig. S4A-C).

When choosing between genital odor from intact males and that from castrated males, *Lmx1b*^{+/+} females preferred intact males over castrated males (Fig. S3B), whereas *Lmx1b*^{+/-} and *Lmx1b*^{-/-} females did not show a preference. The percentage of females preferring intact male genital odor was significantly lower in *Lmx1b*^{-/-} females than either *Lmx1b*^{+/+} or *Lmx1b*^{+/-}

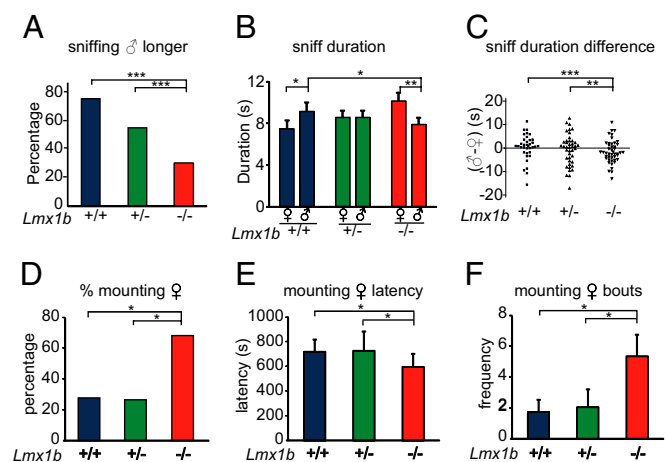


Fig. 2. *Lmx1b*^{-/-} females prefer estrous female genital odor over male genital odor and show more female-female mounting. A test female was presented with a slide smeared with male and estrous female genital excretions. (A-C) $n = 31$ for *Lmx1b*^{+/+} (^{+/+}), $n = 44$ for *Lmx1b*^{+/-} (^{+/-}), $n = 48$ for *Lmx1b*^{-/-} (^{-/-}). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (A) Compared with their *Lmx1b*^{+/+} and *Lmx1b*^{+/-} female littermates, a smaller percentage of *Lmx1b*^{-/-} females sniffed male genital odor longer than estrous female genital odor. (B) *Lmx1b*^{-/-} females preferred estrous female genital odor over male genital odor. Their *Lmx1b*^{+/+} female littermates sniffed male genital odor longer than estrous female genital odor. The sniff duration for male genital odor was shorter in *Lmx1b*^{-/-} females than in their *Lmx1b*^{+/+} female littermates. *Lmx1b*^{+/-} female littermates did not show a preference for either male or female genital odor. (C) Sniff duration of individual females was analyzed: sniff duration for male minus sniff duration for female. (D-F) A test estrous female was analyzed for its mounting of a female (+♀), $n = 24$ for *Lmx1b*^{+/+} (^{+/+}), $n = 15$ for *Lmx1b*^{+/-} (^{+/-}), $n = 19$ for *Lmx1b*^{-/-} (^{-/-}). (D) A higher percentage of female-female mounting occurred in estrous *Lmx1b*^{-/-} females than in their female *Lmx1b*^{+/+} and *Lmx1b*^{+/-} littermates. (E) Female-female mounting latency of *Lmx1b*^{-/-} females was modestly but significantly less than their female *Lmx1b*^{+/+} and *Lmx1b*^{+/-} littermates. (F) Female-female mounting bouts occurred more in *Lmx1b*^{-/-} females than their female *Lmx1b*^{+/+} and *Lmx1b*^{+/-} littermates.

female littermates (Fig. S3C). Thus, *Lmx1b*^{-/-} females were different from the WT females in their preference of odors present in the genital area of intact males.

An intermediate phenotype was also detected in *Lmx1b*^{+/-} mice: they had lost preference between males and females (Fig. 2B) or between intact and castrated males (Fig. S3B). Thus, genital odor preference is also sensitive to the dosage of 5-HT.

When a test mouse was provided with a choice of male odor over saline or female odor over saline, no difference was detected among *Lmx1b*^{-/-}, *Lmx1b*^{+/+}, and *Lmx1b*^{+/-} females (Fig. S5), indicating that *Lmx1b*^{-/-} females were not generally defective in olfaction.

Female-Female Mounting by Mice Lacking Serotonergic Neurons.

When a test female mouse was presented with a target female, 68.4% of estrous *Lmx1b*^{-/-} females mounted female intruders, whereas only ~30% of *Lmx1b*^{+/+} and *Lmx1b*^{+/-} female littermates exhibited this behavior (Fig. 2D). *Lmx1b*^{-/-} females initiated mounting earlier and mounted more frequently than their female littermates (Fig. 2E and F). The mounting behaviors of *Lmx1b*^{-/-} females were similar to the male typical sexual behaviors: they sniffed female mice and tried to grasp the intruder females by the waist before mounting on their back. During mounting, *Lmx1b*^{-/-} females showed pelvic thrusts toward the genital areas of the intruder females. If female intruders escaped from their grasp, *Lmx1b*^{-/-} females often chased them and tried to mount again. This same-sex mounting behavior was not significantly changed by the estrous cycle. Diestrous *Lmx1b*^{-/-} females also showed more mounting behavior than their female littermates (Fig. S6A-C).

When presented with a male mouse, only a small percentage of estrous female mice mounted male intruders. Female-male mounting behavior was not significantly different among *Lmx1b*^{+/+}, *Lmx1b*^{+/-}, and *Lmx1b*^{-/-} females (Fig. S6D-F). Thus, elimination of central serotonergic neurons significantly increased female-female mounting.

Female Sexual Behaviors of Mice Lacking Serotonergic Neurons. To investigate female-typical sexual behaviors, we presented a WT male to a test female. When mounted by males, *Lmx1b*^{-/-} females were similar to *Lmx1b*^{+/+} and *Lmx1b*^{+/-} female littermates: initially showing typical rejection behaviors, such as running and fighting, followed by proceptive and receptive behaviors.

During the first 10 mounts by male mice, *Lmx1b*^{-/-} females showed proceptive behaviors about one time and lordosis behaviors about seven times (Fig. S6G and H). These were not significantly different from their female littermates (Fig. S6G and H). The receptive scores were not significantly different between *Lmx1b*^{-/-} females and their littermates (Fig. S6I). Thus, lack of central serotonergic neurons did not change the female typical sexual behaviors when females encountered males.

Sexual Preference of *Tph2* Knockout Female Mice. Although studies of *Lmx1b* mutants have revealed a role for serotonergic neurons in female sexual preference, it did not show a role for 5-HT. We used mice mutant for tryptophan hydroxylases 2 (*Tph2*), which encodes the enzyme tryptophan hydroxylase required for the first step in the brain's biosynthesis of 5-HT (26). The levels of 5-HT and 5-HIAA were significantly lower in the brains of *Tph2*^{-/-} females than those in *Tph2*^{+/+} or *Tph2*^{+/-} females (Fig. S1C and D). Behavioral analysis of *Tph2*^{-/-} females allowed us to examine the function of 5-HT.

When presented with a WT male and a WT female, *Tph2*^{-/-} females displayed a change in sexual preference. *Tph2*^{+/+} females sniffed males with a shorter latency than females (Fig. S7A), but *Tph2*^{-/-} females did not. *Tph2*^{+/+} females sniffed males longer than females, but *Tph2*^{-/-} females sniffed females longer than males (Fig. 3A). The latency to sniff males was significantly increased in *Tph2*^{-/-} females compared with that in *Tph2*^{+/+} females (Fig. S7A).

The reversal of sexual preference by *Tph2*^{-/-} females was more obvious when comparing the sniffing of the genital and head areas.

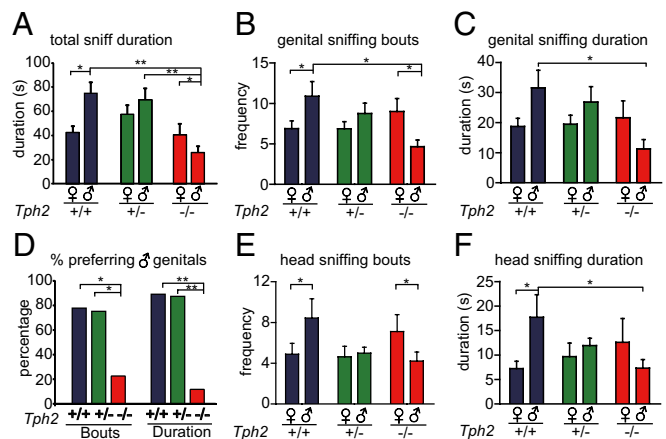


Fig. 3. Sexual preference of *Tph2* knockout female mice. $n = 9$ for *Tph2*^{+/+}, $n = 6$ for *Tph2*^{+/-}, $n = 8$ for *Tph2*^{-/-}. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (A) *Tph2*^{-/-} females sniffed female targets longer than male targets, whereas their *Tph2*^{+/+} littermates sniffed male targets longer than female targets. (B) *Tph2*^{-/-} females sniffed female genitals more frequently than male genitals, whereas their *Tph2*^{+/+} littermates sniffed male genitals more frequently than female genitals. (C) *Tph2*^{-/-} females sniffed male genitals for a shorter time than their *Tph2*^{+/+} littermates. (D) Percentage of mice of each genotype that sniffed male genitals more (bouts) or longer (duration) than female genitals. (E) *Tph2*^{-/-} females sniffed female heads more frequently than male heads, whereas *Tph2*^{+/+} littermates sniffed male heads more. (F) *Tph2*^{-/-} females sniffed male heads shorter than *Tph2*^{+/+} littermates. *Tph2*^{+/+} mice sniffed male heads longer than female heads.

Tph2^{+/+} females sniffed the genital and head areas of males more often than those of females, but *Tph2*^{-/-} females sniffed the genital and head areas of females more than those of males (Fig. 3B and E). Fewer *Tph2*^{-/-} females than their *Tph2*^{+/+} and *Tph2*^{+/-} littermate preferred male over female genital or head areas (Fig. 3E and Fig. S7C and E). The duration of sniffing the genital and head areas of males was reduced in *Tph2*^{-/-} females compared with *Tph2*^{+/+} females (Fig. 3C and F). *Tph2*^{+/-} females often displayed a phenotype between those of *Tph2*^{+/+} and *Tph2*^{-/-} females.

When presented with female and male bedding, more *Tph2*^{-/-} females than *Tph2*^{+/+} and *Tph2*^{+/-} females had a longer duration of staying on the female bedding than male bedding (Fig. S8A). Although *Tph2*^{+/+} and *Tph2*^{+/-} females stayed on male and female bedding for similar durations, *Tph2*^{-/-} females stayed on female bedding for a longer duration than on male bedding (Fig. S8B and C). Thus, *Tph2*^{-/-} also showed preference for females in the bedding choice assay.

Mounting Preference of *Tph2* Knockout Females. When presented with a WT female and a male at the same time, *Tph2*^{-/-} females displayed a strong mounting preference toward the females. *Tph2*^{-/-} females mounted female targets with a shorter latency, higher frequency, and longer duration than the male targets, whereas their littermates showed no mounting preference (Fig. 4A-C).

Approximately 75% of *Tph2*^{-/-} females mounted females, whereas none of their *Tph2*^{+/+} and 37.5% of *Tph2*^{+/-} littermates displayed this behavior (Fig. S7F). *Tph2*^{-/-} females mounted target females with a shorter latency, higher frequency, and longer duration than their littermates (Fig. 4A-C). By contrast, there was no difference between *Tph2*^{-/-} females and their *Tph2*^{+/+} littermates in mounting male targets: the mounting percentage, latency, bouts, and duration were not significantly different (Fig. 4 and Fig. S7F).

Sexual Preference in Serotonergic Defective Mice with Controlled Estrogen Level.

It is known that sex hormones influence sexual behaviors and sexual preference (4, 6, 14, 21, 32). We examined whether 5-HT functioned by changing the levels of sex hormones and found that neither estradiol nor testosterone was significantly

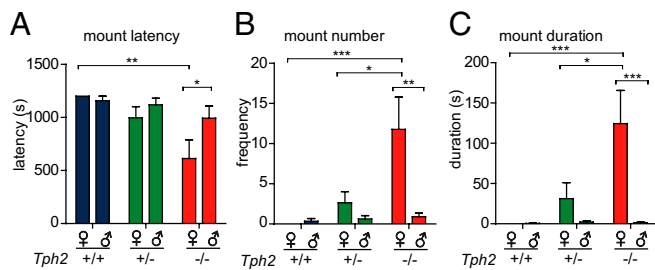


Fig. 4. Mounting preference of *Tph2* mice. $n = 9$ for *Tph2*^{+/+}, $n = 6$ for *Tph2*^{+/-}, $n = 8$ for *Tph2*^{-/-}. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (A) Female–female mounting latency was shorter in *Tph2*^{-/-} females than their *Tph2*^{+/+} littermates. *Tph2*^{-/-} females mounted female targets earlier than male targets. (B) *Tph2*^{-/-} females mounted female targets more than their littermates. *Tph2*^{-/-} females mounted female targets more frequently than male targets. (C) *Tph2*^{-/-} females mounted female targets longer than their littermates. *Tph2*^{-/-} females mounted female targets longer than male targets.

different among estrous or diestrous *Tph2*^{-/-}, *Tph2*^{+/-}, and *Tph2*^{+/+} females (Fig. S9), suggesting that it is unlikely that 5-HT regulates sexual preference by controlling sex hormones.

We ovariectomized either *Tph2*^{+/+} or *Tph2*^{-/-} females and restored their estradiol to the same level by injection (OVX+E). After such treatments, *Tph2*^{-/-} females still displayed strong preference for females over males. They sniffed female genitals earlier, more frequently, and longer than male genitals (Fig. 5A and Fig. S10A and B). More *Tph2*^{-/-} females sniffed female heads or genitals longer and more frequently than their *Tph2*^{+/+} littermates (Fig. 5B and D and Fig. S10C and D). *Tph2*^{-/-} females had a longer latency to sniffed male genitals than their *Tph2*^{+/+} littermates (Fig. S10A), and they sniffed male genitals less frequently and with shorter duration than their *Tph2*^{+/+} littermates (Fig. 5A and Fig. S10B). *Tph2*^{-/-} also sniffed male heads less frequently and for a shorter duration (Fig. 5C and Fig. S10F). *Tph2*^{-/-} sniffed female genitals more frequently and with a longer duration (Fig. 5A and Fig. S10B).

When presented with a female alone, a higher percentage of *Tph2*^{-/-} females than *Tph2*^{+/+} females mounted females (Fig. S10G). *Tph2*^{-/-} females mounted females faster, more frequently, and longer than their *Tph2*^{+/+} littermates (Fig. 5E and F and Fig. S10H).

Requirement of 5-HT in Adult Females for Sexual Preference. Because *Lmx1b*^{-/-} and *Tph2*^{-/-} mice lacked central serotonergic neurons or 5-HT from embryogenesis to adulthood, it was unclear whether the phenotype in sexual preference was caused indirectly by developmental defects or directly by involvement of 5-HT in adult behaviors. We first used *p*-chlorophenylalanine (pCPA), a Tph inhibitor, to pharmacologically deplete 5-HT from WT adult animals (33).

pCPA significantly lowered the levels of 5-HT and 5-HIAA in the adult brain (Fig. S1E and F). In the mating choice assay, we found that 5-HT depletion significantly decreased the percentage of female mice preferring male over female genitals (Fig. 6A and Fig. S11F and G). 5-HT depletion increased the latency of females to sniff male genitals, but did not change their latency to sniff female genitals (Fig. S11E). Control females injected with saline sniffed male genitals more frequently (Fig. 6B) and for a longer duration than female genitals (Fig. 6C). By contrast, female mice injected with pCPA sniffed female genitals more frequently and for a longer duration than male genitals (Fig. 6B and C), resulting in a reversal of preference of sexually dimorphic odors (Fig. S11F and G). 5-HT depletion also changed female preference in sniffing heads and the whole body (Fig. S11A–D).

When a female was tested with another female, 5-HT depletion also significantly increased the percentage of females with female–female mounting behavior (Fig. S11H). Latency for female–female

mounting was decreased (Fig. S11I) and frequency was increased by 5-HT depletion (Fig. S11J).

Results with pCPA depletion in adults indicate that 5-HT functions in adulthood to regulate sexual preference of females.

Because pCPA can have nonspecific effect (34), we carried out genetic rescue experiments in adult females to demonstrate a role for 5-HT in adulthood. Injection of 5-HTP, an intermediate of 5-HT synthesis downstream of *Tph2*, into adult mice rescued the phenotype of *Tph2*^{-/-} females in olfactory preference (Fig. 6D–G and Fig. S12A and B), female–female mounting (Fig. 6H and I), and bedding preference (Fig. S12C and D), further supporting a role for 5-HT in the preference behavior of adults.

Discussion

Our findings with *Lmx1b*^{-/-} mice, *Tph2*^{-/-} mice, and mice treated with pCPA have led to the conclusion that serotonergic signaling is involved in controlling sexual preference in adult females.

This article has extended significantly beyond the male sexual preference article in three aspects (26). (i) A genetic alternation made in the laboratory has reversed sexually preference without changing sex hormone levels. Previous studies in *Drosophila* and mammals have found genetic mutations causing an increased male–male (22) or female–female (13, 25) sexual activity, but none of these mutations has been shown to cause a reversal of preference in a behavioral assay. *Lmx1b*^{-/-} mutant females studied here are different from *AFP*^{-/-} females reported previously (13) in that the *Lmx1b*^{-/-} females are as receptive as WT females to males when presented only with male partners. Our findings with *Lmx1b*^{-/-} mutant females indicate that it is possible to observe same-sex preference in the laboratory as well as in the wild (13, 25). (ii) In the male sexual preference article, there is an important issue as to whether 5-HT only plays a role in inhibiting sexual drive or whether it has an additional role in sexual preference. It is possible that an overall increase in sexuality in serotonergic mutants can appear as more increase for male–male sexual activity in

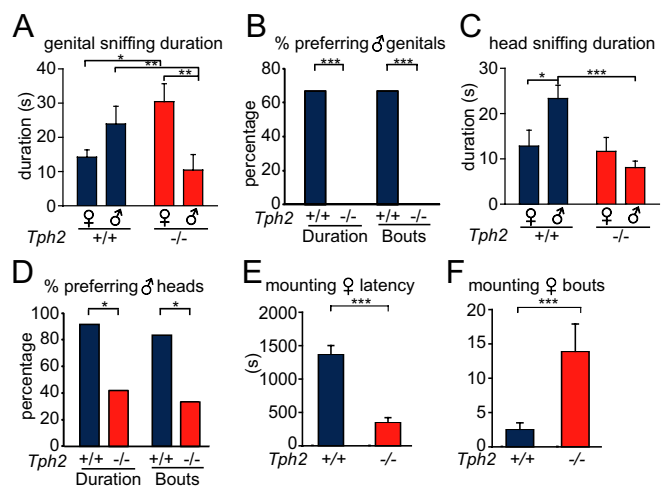


Fig. 5. Sexual preference of estradiol treated ovariectomized (OVX+E) females. $n = 12$ for OVX+E *Tph2*^{+/+}, $n = 12$ for OVX+E *Tph2*^{-/-}. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (A) OVX+E *Tph2*^{-/-} females sniffed female genitals longer than male genitals. OVX+E *Tph2*^{-/-} sniffed female genitals longer than their OVX+E *Tph2*^{+/+} littermates. OVX+E *Tph2*^{-/-} sniffed male genitals shorter than their OVX+E *Tph2*^{+/+} littermates. (B) Percentage of mice of each genotype that sniffed male genitals more (bouts) or longer (duration) than female genitals. (C) OVX+E *Tph2*^{-/-} females sniffed male heads shorter than their littermates. OVX+E *Tph2*^{+/+} females sniffed male heads longer than female heads, whereas OVX+E *Tph2*^{-/-} females did not display preference. (D) Percentage of mice of each genotype that sniffed male heads more (bouts) or longer (duration) than female heads. (E) OVX+E *Tph2*^{-/-} mounted female targets earlier than their OVX+E *Tph2*^{+/+} littermates. (F) *Tph2*^{-/-} females mounted female targets more frequently than their *Tph2*^{+/+} littermates.

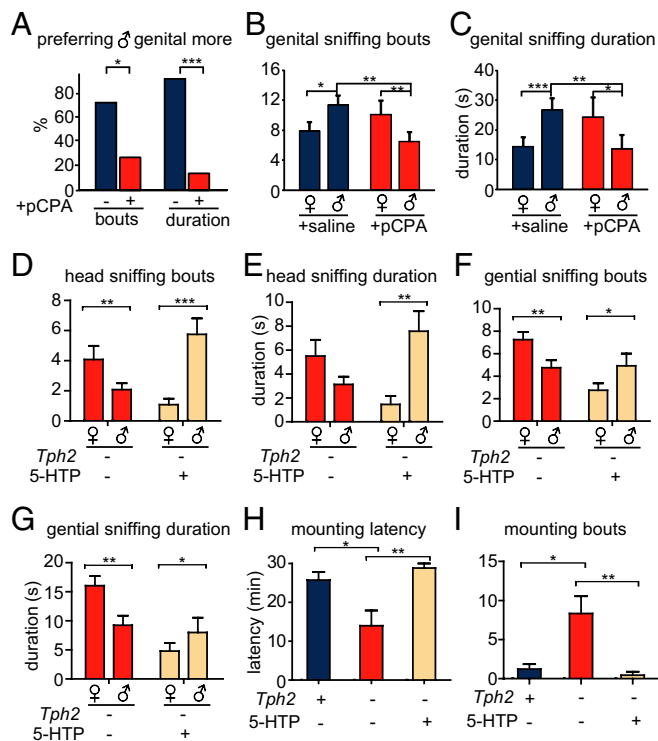


Fig. 6. Sexual preference of adult females after pCPA or 5-HTP injection. (A–C) Each adult C57BL/6J female was treated with saline (+saline, $n = 15$) or pCPA (+pCPA, $n = 15$) and presented with a male and female target. (D–G) Twelve $Tph2^{-/-}$ females (labeled as – for $Tph2$) were treated with 5-HTP (indicated as + for 5-HTP treatment), 12 $Tph2^{-/-}$ females were treated with saline. (H and I) $Tph2^{+/+}$ were treated with saline ($n = 9$). $Tph2^{-/-}$ were treated with saline ($n = 9$) or 5-HTP ($n = 9$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (A) pCPA decreased the percentage of females sniffing male genitals more frequent or longer than female genitals. (B) pCPA reversed the preference of females in genital sniffing bouts. Male genital sniffing bouts was significantly decreased in pCPA treated females than in control females. (C) pCPA reversed the preference of females in genital sniffing duration. Male genital sniffing duration was significantly decreased in pCPA treated females than in control females. (D and E) Injection of 5-HTP could rescue the same-sex preference in head sniffing bouts and duration of $Tph2^{-/-}$ females. (F and G) Injection of 5-HTP could rescue the same sex preference in genital sniffing bouts and duration of $Tph2^{-/-}$ females. The phenotype of head sniffing latency and genital sniffing latency was more variable and not as informative as those of sniffing frequency and duration. (H and I) Injection of 5HTP rescued the female–female mounting phenotype of $Tph2^{-/-}$ females.

mutants because, with the baseline lower for male–male activity than that for male–female activity, an overall increase in both male–male and male–female activities can lead to more increase in male–male than male–female activity, thus appearing as a loss of sexual preference in the male mutants. In the present article, our finding of a reversal in sexual preference in female mutants clearly establishes a role for 5-HT in sexual preference because hypersexuality is very difficult to explain the reversal of sexual preference. (iii) Although our previous studies of male mutants did not detect a role for 5-HT in pheromone sensing in the periphery (26), it could not be completely ruled out that loss of sexual preference in males could be attributed to defective olfactory processing of innate sexual signals (35). However, the reversal of sexual preference observed in female mutants cannot be easily explained by a defective peripheral olfactory sensing and is more consistent with a central mechanism of 5HT in controlling sexual preference.

Sexual behaviors between members of the same sex have been observed after 5-HT depletion (36–42), but none of the previous

studies has demonstrated a reversal of sexual preference. In fact, those experiments were carried out to investigate the control of sexual activities, not the regulation of sexual preference. Male crickets showed high-level courtship behavior to male crickets 7 d after antenna removal, when 5-HT was very low (43). Central 5-HT level also modulates sexual behaviors in ovariectomized *Cnemidophorus uniparens* (44, 45). 5-HT depletion causes male–male mounting in cats, rats, and rabbits (37, 41). Female rats also mounted female intruders after 5-HT depletion (42). Previous reports have interpreted the phenotype as hypersexuality and concluded that 5-HT inhibits male and female sexual activity. In $Lmx1b^{-/-}$ mutant females, no general increase in sexual activities has been observed: their receptivity toward males was similar to WT littermates when presented only with males. Rather, they showed increased activities toward females and decreased activities toward males when given a choice of a male and a female. Our results demonstrate that lack of central serotonergic neurons or 5-HT causes a reversal of sexual preference, revealing a role for 5-HT in regulating sexual preference.

There are 14 5-HT receptors distributed in different regions of the mouse brain. It remains to be determined which are involved in sexual preference. It will also be important to study signaling downstream of 5-HT.

Materials and Methods

Animals. $Lmx1b^{-/-}$, $Lmx1b^{floxpl/floxpl}$, $ePet-Cre$, and $Tph2^{-/-}$ mice were generated and genotyped as previously described (26, 27). All mice were maintained on a 12D:12L cycle with food and water ad libitum. All test mice were individually housed, 12- to 16-wk-old females. $Lmx1b^{+/+}$ mice include $Lmx1b^{+/+}/ePet-Cre^{+}$, $Lmx1b^{floxpl/floxpl}/ePet-Cre^{-}$, and $Lmx1b^{floxpl/+}/ePet-Cre^{-}$ mice, which behaved similarly. Castrated males and ovariectomized females were used at least 2 wk after surgery. All behavioral assays were carried out 2–4 h after light was turned off and analyzed in a double-blind manner. Animal experiments have been approved by the Animal Review Board of Peking University.

Mating Choice Assay. This assay was carried out essentially as previously described (22). Briefly, a male and an estrous female C57BL/6J mouse were placed into the home cage of the test mouse. Behaviors of the test female were recorded for 20 min and analyzed. To reduce the influence of the male intruders, we used castrated males swabbed with urine from an intact male on the genital area (80 μ L) and back (20 μ L). Contacts initiated by test mice using the snout were recorded as sniff.

Genital Odor Preference Assay. A glass slide was smeared with the genitals of two donors, each on one side of the slide. The slide was clamped at the middle, which was clean, and hung in the middle of the home cage of the test mouse. Behaviors of the test mice were recorded for 3 min and analyzed. Sniff was recorded when test mice contacted the slide with the snout.

Bedding Preference Assay. Bedding from group-housed adult C57BL/6J males or females were not changed for 4 d. Ten grams of male or female bedding were put in one side on the bottom of a cage in an area of 11.5 \times 17 cm^2 . The male and female beddings were prevented from mixing by a plastic bar of 6 cm. The size of cage was 29 \times 17 \times 15 cm (L \times W \times H). A grid of plastic bars separated the test mice from the bedding on the bottom of the cage. The bars were 5 mm wide with 5-mm intervals. The test mouse was put into the cage to be familiarized with the cage without bedding for 5 min before the mice were taken out and the bedding and a clean grid was put into the cage. After each assay, the cage was washed with water and then alcohol to remove odor.

Resident-Intruder Assay. A C57BL/6J mouse was placed into the home cage of a test female. Behaviors of the test female were recorded for 30 min and analyzed. Two types of mice were used separately as the intruder: an intact female or a castrated male swabbed with urine from an intact male.

Lordosis. This assay was carried out and analyzed essentially as previously described (46, 47). Ten-week-old sex-experienced C57BL/6J males were individually housed for 1 wk before being used. A vaginal smear was obtained from the test female 2–3 h before light-off every day. Test females were in estrous. A test female was placed into the home cage of a male. Behaviors of the test female were recorded and scored during 10 mounts exhibited by the male resident and analyzed as previously described (46). Briefly,

unreceptive female behavior was defined as rearing, kicking, or fleeing response to mounting (score 0). Proceptive behavior was defined as the still posture of the female mice without dorsiflexion of vertebral column during mounting. Dorsiflexion of female vertebral column during mounting was recorded as receptive behavior (score 1–3 with 0.5 intervals as previously described in ref. 39). If the male exhibited less than 10 mounts during 15 min, the result was excluded from analysis.

Pharmacological Treatment. Ten-week-old naive C57BL/6J females were used. pCPA (400 mg/kg body weight in saline, SIGMA-Aldrich) was injected intraperitoneally once per day for 4 d. Control mice were injected with saline. Behavioral assays were carried out on the fifth day.

For OVX+E females, estradiol (200 μ g/kg body weight) (42) was injected s.c. daily after surgery. Resident-intruder assays were carried out 2 wk after surgery. Mating choice assays were done 1 wk later.

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Supporting Information

Zhang et al. 10.1073/pnas.1220712110

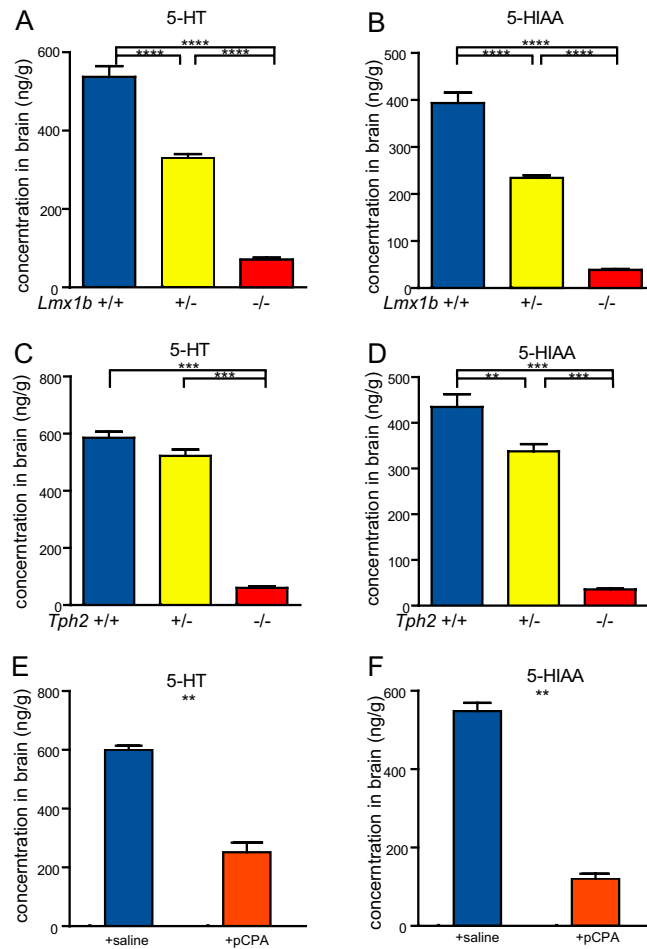


Fig. S1. Levels of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the brain. (A) 5-HT in the brains of *Lmx1b*^{+/+} (^{+/+}, *n* = 7), *Lmx1b*^{+/-} (^{+/-}, *n* = 7), and *Lmx1b*^{-/-} (^{-/-}, *n* = 7) female mice were analyzed by HPLC. (B) 5-HIAA in the brains of *Lmx1b*^{+/+}, *Lmx1b*^{+/-}, and *Lmx1b*^{-/-} females. (C) 5-HT in the brains of *Tph2*^{-/-} (*n* = 7), *Tph2*^{+/-} (*n* = 8), and *Tph2*^{+/+} (*n* = 7) females. (D) 5-HIAA in the brains of *Tph2*^{-/-}, *Tph2*^{+/-}, and *Tph2*^{+/+} females. (E) 5-HT in the brains of female mice injected with saline (*n* = 5) or *p*-chlorophenylalanine (pCPA) (*n* = 6). (F) 5-HIAA in the brains of female mice injected with saline or pCPA. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

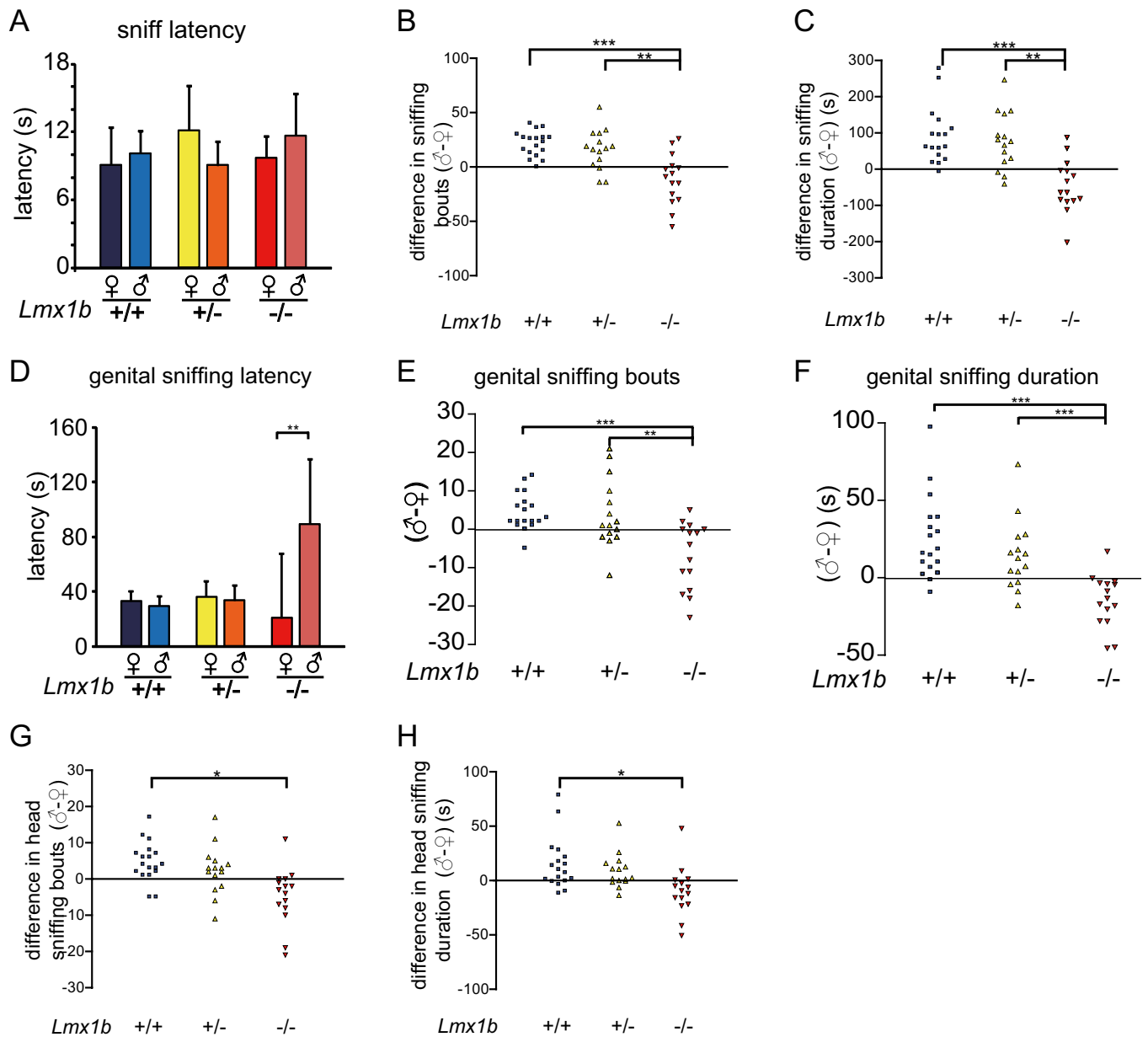


Fig. S2. Sexual preference of females in the mating choice assay. $n = 18$ for $Lmx1b^{+/+}$ ($+/+$), $n = 15$ for $Lmx1b^{+/-}$ ($+/-$), $n = 15$ for $Lmx1b^{-/-}$ ($-/-$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (A) Sniff latencies of the whole body were not different among $Lmx1b^{-/-}$ females and their $Lmx1b^{+/+}$ or $Lmx1b^{+/-}$ female littermates. (B) Difference in sniffing bouts analyzed in individual females. (C) Difference in sniffing duration analyzed in individual females. (D) The latency to sniff male genital was lengthened in $Lmx1b^{-/-}$ females; thus, they sniffed female genitals first, whereas their female littermates did not show sexual preference in genital sniffing latency. (E) Genital sniffing bouts were analyzed by differences of each individual female: its bouts for sniffing male minus its bouts for sniffing female. (F) Genital sniffing duration was analyzed in individual females: its duration of sniffing males minus its duration of sniffing females. (G) Difference in sniffing head bouts analyzed in individual females. (H) Difference in sniffing head duration analyzed in individual females.

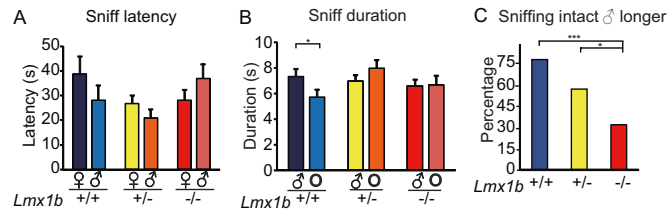


Fig. S3. *Lmx1b*^{-/-} females prefer intact over castrated male genital odor. (A) Data are from the same experiments as those in Fig. 2. Latencies of females of different genotype to sniff male or female genital odor were not statistically different. (B and C) *n* = 32 for *Lmx1b*^{+/+}, *n* = 36 for *Lmx1b*^{+/-}, *n* = 40 for *Lmx1b*^{-/-}. (B) *Lmx1b*^{+/+} females sniffed intact male genital odor longer than castrated male genital odor (o); *Lmx1b*^{+/-} and *Lmx1b*^{-/-} females did not show preference between genital odors of intact males and castrated males. (C) Compared with their *Lmx1b*^{+/+} and *Lmx1b*^{+/-} female littermates, a smaller percentage of *Lmx1b*^{-/-} females sniffed intact male genital odor longer than castrated male genital odor.

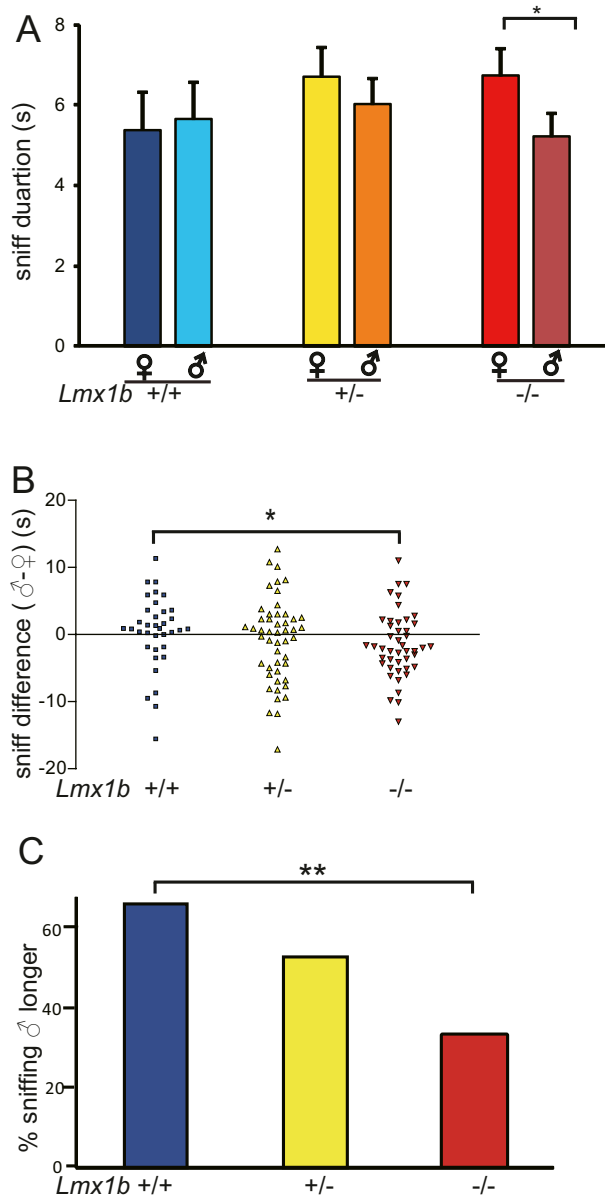


Fig. S4. Genital odor preference of females. A test female was presented with a slide smeared with male genital excretion and diestrous female genital excretion. *n* = 36 for *Lmx1b*^{+/+} (+/+), *n* = 49 for *Lmx1b*^{+/-} (+/-), *n* = 45 for *Lmx1b*^{-/-} (-/-). **P* < 0.05, ***P* < 0.01. (A) *Lmx1b*^{-/-} females preferred the genital odor of diestrous females over that of males. (B) Analysis of difference in sniff duration in females. (C) Percentage of females sniffing male genital odor longer than that of diestrous female.

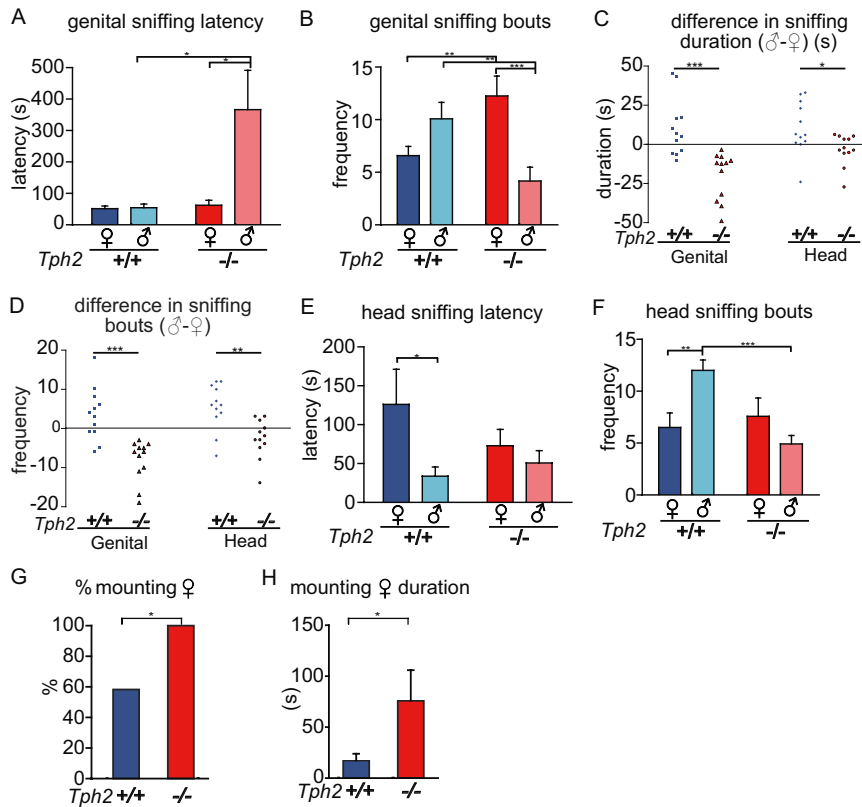


Fig. S10. Sexual preference of estradiol-treated ovariectomized (OVX+E) females. Data are from the same experiments as those in Fig. 5. (A) OVX+E *Tph2*^{-/-} females sniffed female genital area earlier than male genital area. OVX+E *Tph2*^{-/-} sniffed male genitals later than their OVX+E *Tph2*^{+/+} littermates. (B) OVX+E *Tph2*^{-/-} females sniffed female genitals more frequently than male genitals. OVX+E *Tph2*^{-/-} sniffed female genitals more frequently than their OVX+E *Tph2*^{+/+} littermates. OVX+E *Tph2*^{-/-} sniffed male genitals less often than their OVX+E *Tph2*^{+/+} littermates. (C) Genital or head sniffing duration was analyzed in individual females as the duration of sniffing males minus the duration of sniffing females. (D) Genital or head sniffing bouts were analyzed in individual females as the bouts of sniffing males minus the bouts of sniffing females. (E) OVX+E *Tph2*^{+/+} females sniffed male heads earlier than female heads, whereas OVX+E *Tph2*^{-/-} female mice did not show preference. (F) OVX+E *Tph2*^{-/-} females sniffed male heads less than their littermates. OVX+E *Tph2*^{+/+} females sniffed male heads more than female heads, whereas OVX+E *Tph2*^{-/-} females did not display preference. (G) A higher percentage of female–female mounting occurred in OVX+E *Tph2*^{-/-} females than that in their OVX+E *Tph2*^{+/+} littermates. (H) *Tph2*^{-/-} females mounted female targets longer than their *Tph2*^{+/+} littermates.

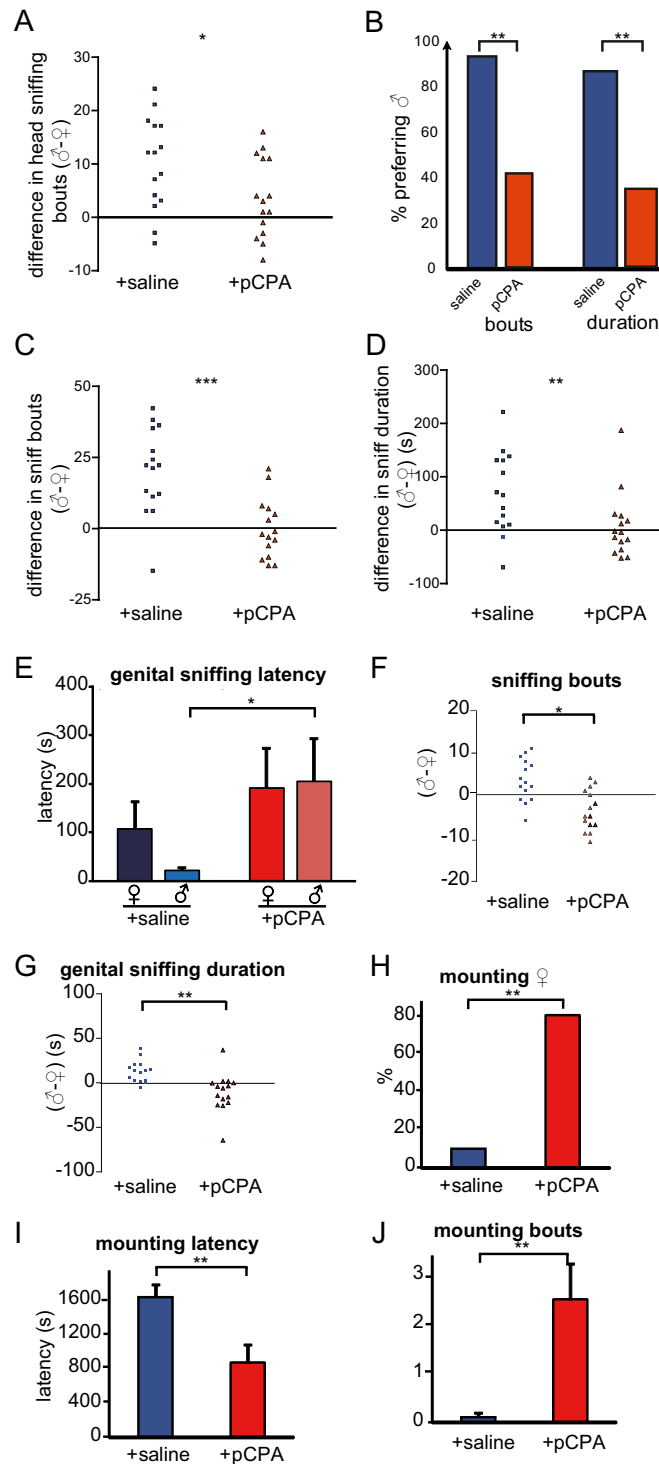


Fig. S11. (A–D) Sexual preference of females treated with pCPA. (A–G) Each C57BL/6J female treated with either saline (+saline, $n = 15$) or pCPA (+pCPA, $n = 15$) was presented with a male and a female target mouse. (H–J) Each adult C57BL/6J female was treated with saline ($n = 10$) or pCPA ($n = 10$) and presented with a female. (A) The preference for male heads in sniffing bouts by females was significantly reduced by pCPA. (B) The percentage of female preferring (the whole body of) males in bouts or duration was significantly reduced by pCPA. (C) Analysis of difference in sniff bouts of individual females. (D) Analysis of difference in sniff duration of individual females. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (E) The latency for sniffing male genitals is significantly later in pCPA-treated females than in control females. (F and G) Analysis of differences in genital sniffing bouts and duration in individual females. (H–J) pCPA-treated females fiercely mounted females. (H) pCPA increased the percentage of females mounting intruder females. pCPA decreased mounting latency (I) and increased mounting bouts (J) of females. Data are from the same experiments as those in Fig. 6.

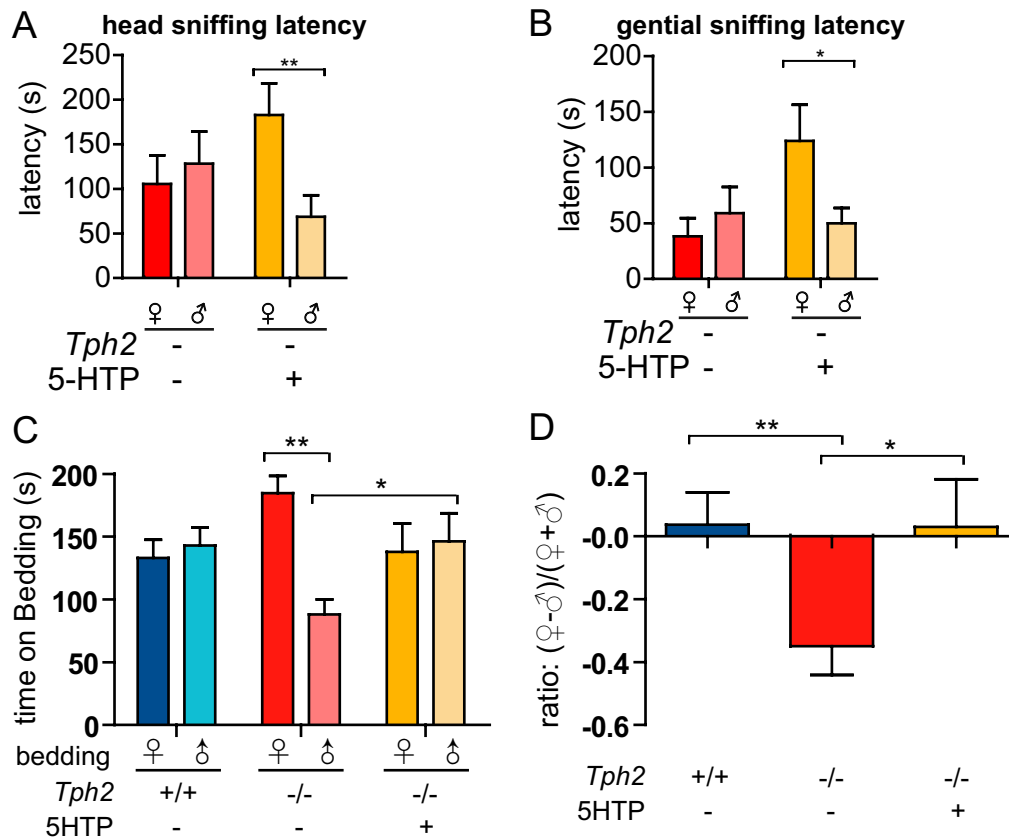


Fig. S12. 5-HTP rescue of sexual preference of adult females in sniff latency and bedding preference. (A and B) Data are from the same experiments as those in Fig. 6 H–K. (A) Injection of 5-HTP rescued the same-sex preference in head-sniffing latency of *Tph2*^{-/-} females. (B) Injection of 5-HTP could rescue the same-sex preference in genital sniffing latency of *Tph2*^{-/-} females. (C and D) Bedding preference of females treated with 5-HTP. *Tph2*^{+/+} females were treated with saline (*n* = 11). *Tph2*^{-/-} females were treated with saline (*n* = 12) or 5-HTP (*n* = 12). **P* < 0.05, ***P* < 0.01. (C) *Tph2*^{-/-} females strongly preferred female over bedding. 5HTP rescued the bedding preference of *Tph2*^{-/-} females. (D) Compared with *Tph2*^{+/+}, a higher percentage of *Tph2*^{-/-} females spent more time above female bedding than male bedding. 5HTP rescued the bedding ratio of *Tph2*^{-/-}.