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

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

\textsuperscript{a}State 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 \textsuperscript{b}National 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 (TrpC2), which encodes a cation channel in the vomeronasal organ (22, 23), or cyclic nucleotide-gated channel α2 (Cgna2) in the main olfactory epithelium (24) resulted in loss of sexual preference in male mice. TrpC2–/– 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\textsuperscript{−/−}) could be generated by crossing Cre recombinase driven by Pet-1 enhancer (ePet-Cre) into Lmx1b\textsuperscript{loxP/loxP} mice, leading to the absence of serotonergic neurons in the brain without affecting 5-HT in the periphery (27). We have obtained female Lmx1b\textsuperscript{−/−} mice female targeted that the male of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were lower in the brains of homozygous (Lmx1b\textsuperscript{−/−}) mutant females than those in the WT (Lmx1b\textsuperscript{+/+}) and heterozygous (Lmx1b\textsuperscript{+/−}) females (Fig. S1 A 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\textsuperscript{−/−} females showed the opposite preference. The latency for sniffing target mice was not different among the Lmx1b\textsuperscript{+/+}, Lmx1b\textsuperscript{+/−}, and Lmx1b\textsuperscript{−/−} females (Fig. S2 A). When the duration of sniffing the whole body was compared, Lmx1b\textsuperscript{+/−} and Lmx1b\textsuperscript{−/−} females spent longer time sniffing male target mice than female targets (Fig. 1A and Fig. S2C). By contrast, Lmx1b\textsuperscript{−/−} females sniffed female targets longer than male targets (Fig. 1A and Fig. S2C).

In sniff bouts, Lmx1b\textsuperscript{+/−} and Lmx1b\textsuperscript{−/−} females showed preference for males over females, whereas Lmx1b\textsuperscript{+/−} 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\textsuperscript{−/−} mice showed a shorter latency to approach and sniff the genital area of female targets. It was clear that WT females preferred male 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).

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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\textsuperscript{−/−} mice showed a shorter latency to approach and sniff the genital area of female targets than that of male targets, whereas Lmx1b\textsuperscript{+/+} or Lmx1b\textsuperscript{+/−} females did not (Fig. S2D). Lmx1b\textsuperscript{−/−} female littersmates 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\textsuperscript{+/−} females (Fig. 1B and C and Fig. S2 E and F); they sniffed the female genital area more frequently and with longer duration than the male genital area. Compared with their Lmx1b\textsuperscript{+/+} and Lmx1b\textsuperscript{−/−} littersmates, a significantly smaller percentage of Lmx1b\textsuperscript{−/−} 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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Neurons showed preference for females over males. This suggests that this phenotype is sensitive to the dosage of 5-HT.

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Fig. 1. Lmx1b<sup>−/−</sup> female mice preferred female over male mice. A test female was presented with a male and a female target. n = 18 for Lmx1b<sup>−/−</sup> (+/+), n = 15 for Lmx1b<sup>−/−</sup> (+/−), n = 4.5 for Lmx1b<sup>−/−</sup> (−/−). P < 0.05, **P < 0.01, ***P < 0.001. (A) Lmx1b<sup>−/−</sup> females sniffed females longer than males, whereas their Lmx1b<sup>−/−</sup> and Lmx1b<sup>−/−</sup> female littermates sniffed males for a shorter time than their female littermates. Lmx1b<sup>−/−</sup> female mice sniffed females longer than their Lmx1b<sup>−/−</sup> littermates. (B) Lmx1b<sup>−/−</sup> females sniffed female genitals more often than male genitals, whereas their Lmx1b<sup>−/−</sup> female littermates sniffed male genitals more often than female genitals. Lmx1b<sup>−/−</sup> female littermates did not show sexual preference in sniff bouts. Lmx1b<sup>−/−</sup> females sniffed male genitals less and female genitals more than Lmx1b<sup>−/−</sup> females. (C) Lmx1b<sup>−/−</sup> females sniffed female genitals longer than male genitals, whereas their Lmx1b<sup>−/−</sup> and Lmx1b<sup>−/−</sup> female littermates sniffed male genitals longer than female littermates. There is no significant difference among Lmx1b<sup>−/−</sup>, Lmx1b<sup>+/−</sup>, and Lmx1b<sup>+/+</sup> females in the duration of sniffing female genitals, but Lmx1b<sup>−/−</sup> 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<sup>−/−</sup> females sniffed female heads more than male heads, whereas their Lmx1b<sup>+/−</sup> female littermates sniffed male heads more. Lmx1b<sup>−/−</sup> females had an intermediate phenotype: they had no preference for either males or females. (F) Lmx1b<sup>−/−</sup> females sniffed female heads for a longer duration than male heads, whereas their Lmx1b<sup>+/−</sup> and Lmx1b<sup>−/−</sup> female littermates sniffed male heads longer than female heads.

An intermediate phenotype was detected in Lmx1b<sup>−/−</sup> females: they were similar to Lmx1b<sup>+/−</sup> females in sniff duration preference (Fig. 1 A, C, and F and Fig. S2F), but their preference was not statistically significant when the preference was analyzed with sniff bout frequency (Fig. 1 B and E). This is consistent with an intermediate level of 5-HT in Lmx1b<sup>−/−</sup> females (Fig. 4 D), 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 phenomoral 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<sup>−/−</sup> and Lmx1b<sup>−/−</sup> females sniffed genital odor from males longer than from that of estrous females (Fig. 2A), whereas a significantly lower percentage of Lmx1b<sup>−/−</sup> females sniffed male genital odor longer than female genital odor (Fig. 2A). Lmx1b<sup>−/−</sup> females sniffed male genital odor longer than female genital odor (Fig. 2B). Lmx1b<sup>+/−</sup> females sniffed male and female genital odor equivalently (Fig. 2B). Lmx1b<sup>−/−</sup> females sniffed female genital odor longer than male genital odor (Fig. 2B). Lmx1b<sup>−/−</sup> 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<sup>−/−</sup> females were significantly different from Lmx1b<sup>+/−</sup> and Lmx1b<sup>−/−</sup> female littermates (Fig. S3A). When choosing between genital odors from diestrous females and males, Lmx1b<sup>−/−</sup> females also showed same-sex preference, whereas Lmx1b<sup>+/−</sup> and Lmx1b<sup>−/−</sup> females showed no preference (Fig. S4 A–C).

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

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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–Male Mounting by Mice Lacking Serotonergic Neurons.

When a test female mouse was presented with a target female, 68.4% of estrous $Lmx1b^{-/-}$ females mounted male 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. When intruder intruders escaped from the genital area of intact males, $Lmx1b^{-/-}$ 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–male mounting.

Female Sexual Behaviors of Mice Lacking Serotonergic Neurons.

To investigate female-atypical 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 preceptive and receptive behaviors.

During the first 10 mounts by male mice, $Lmx1b^{-/-}$ females showed preceptive behaviors about one time and lordosis behaviors about seven times (Fig. S6 G and H). These were not significantly different from their female littermates (Fig. S6 G and H). The receptive scores were not significantly different between $Lmx1b^{-/-}$ females and their littermates (Fig. S6d). 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. S1 C 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.
After such treatments, Lmx1b (Fig. 5) females to sniff male genitals, but did not change their latency
A
with saline sniffed male genitals more frequently (Fig. 6
A
and Fig. S10
A
and B), female–female mounting (Fig. 6 H and I), and bedding preference (Fig. S12 C 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 serotonin 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 ovariocistomized (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 their OVX+E Tph2−/− littersmates. OVX+E Tph2−/− sniffed male genitals shorter than their OVX+E Tph2+/− littersmates. (B) Percentage of mice of each genotype that sniffed male heads more (bouts) or longer (duration) than female heads. (C) 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+/− littersmates. (F) Tph2−/− females mounted female targets more frequently than their Tph2+/− littersmates.

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. 6 D–G and Fig. S12 A and B), female–female mounting (Fig. 6 H and I), and bedding preference (Fig. S12 C and D), further supporting a role for 5-HT in the preference behavior of adults.

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 adulthood. 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. S1 E 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. 6 A and Fig. S11 F 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. S11 E). Controls females injected with saline sniffed male genitals more frequently (Fig. 6 B) and for a longer duration than female genitals (Fig. 6 C). By contrast, female mice injected with pCPA sniffed female genitals more frequently and for a longer duration than male genitals (Fig. 6 B and C), resulting in a reversal of preference of sexually dimorphic odors (Fig. S11 F and G). 5-HT depletion also changed female preference in sniffing heads and the whole body (Fig. S11 A–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. S11 F). Latency for female–female mounting was decreased (Fig. S11 I) and frequency was increased by 5-HT depletion (Fig. S11 D).

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 ovariocistomized 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 genitlal cells earlier, more frequently, and longer than male genitals (Fig. 5 A and Fig. S10 A and B). More Tph2−/− females sniffed female heads or genitals longer and more frequently than their Tph2+/− littersmates (Fig. 5 B and D and Fig. S10 C and D). Tph2+/− females had a longer latency to sniff male genitals than their Tph2−/− littersmates (Fig. S10 A), and they sniffed male genitals less frequently and with shorter duration than their Tph2−/− littersmates (Fig. S10 B and Fig. S10 B). Tph2+/− also sniffed male heads less frequently and for a shorter duration (Fig. 5 C and Fig. S10 F). Tph2−/− sniffed female genitals more frequently and with a longer duration (Fig. 5 A and Fig. S10 F).

When presented with a female alone, a higher percentage of Tph2−/− females than Tph2+/+ females sniffed females (Fig. S10 G). Tph2−/− females mounted females faster, more frequently, and longer than their Tph2+/− littersmates (Fig. 5 E and F and Fig. S10 H).

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−/− littersmates. Tph2−/− females sniffed female targets earlier than male targets. (B) Tph2−/− females sniffed female targets more frequently than their littermates. (C) Tph2−/− females sniffed male genitals more frequently and for a longer duration than male targets. (D) Tph2−/− females sniffed male targets longer than their littermates. (E) Tph2−/− females sniffed male targets longer than male targets.
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 unilateral 5-HT1a knockdown in the ventral horn (43). Central 5-HT level also modulates sexual behaviors in ovariectomized C. pennipes (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 serotoninergic 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−/−, Lmx1blox/lox; ePet-Cre, and Tph2−/− mice were generated and genotyped as previously described (26, 27). All mice were maintained on a 12:12 h cycle with food and water ad libitum. All test mice were individually housed, 12- to 16-wk-old females. Lmx1b−/− mice include Lmx1blox/lox;ePet-Cre−, Lmx1blox/lox;ePet-Cre+ and Lmx1blox/lox;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 μL) and back (20 μL). Contacts initiated by test mice using the snout were recorded as sniff.

Genital Odor Preference Assay. A glass slide was smeared with the genitils 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 mouse 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 × 17 cm². The male and female beddings were prevented from mixing by a plastic bar of 6 cm. The size of cage was 29 × 17 × 15 cm (L × W × 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 estrus. A test female was placed into the home cage of a male. Behaviors of the test male 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 O VX + E females, estradiol (200 μg/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.


Statistics. Data are presented as means ± SEM in all bar graphs. Behavioral data were analyzed using nonparametric Kruskal-Wallis or Mann Whitney U-tests. Dunn’s comparison was used if Kruskal-Wallis test was significant. Percentages were analyzed using Fisher’s exact test. ELISA and immunohistochemistry results were analyzed using the Wilcoxon signed-rank test. Significance was set as P < 0.05.

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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. S5. Odor preference of female mice. n = 7 for LmX1b+/+, n = 5 for LmX1b+/-, n = 5 for LmX1b-/-.

(A–C) When a female mouse was presented with a choice of female genital odor and saline, it preferred female genital odor over saline. This was not affected by the genotype of LmX1b. (D–F) All test female mice, regardless of their LmX1b genotype, preferred male genital odor over saline.
Fig. S6. Sexual behaviors of Lmx1b+/− females. *P < 0.05, **P < 0.01, ***P < 0.001. (A–C) Diestrous Lmx1b+/− females mounted WT females. A diestrous female was presented with a target WT female and female–female mounting was analyzed. (A) A higher percentage of diestrous Lmx1b+/− females (n = 34) than their Lmx1b+/+ (n = 19) diestrous littermates mounted WT target females. (B) Female mounting latency was shorter for Lmx1b+/− diestrous females than their Lmx1b+/+ and Lmx1b−/− littermates. (C) Female mounting frequency of diestrous Lmx1b+/− females was higher than their diestrous female Lmx1b+/+ and Lmx1b−/− littermates. (D–F) A test female was analyzed for its mounting of a male (+/−), n = 11 for Lmx1b+/+, n = 18 for Lmx1b−/−, n = 23 for Lmx1b−−. (G–I) A test female was presented with a male, n = 27 for Lmx1b+/+, n = 8 for Lmx1b+/−, n = 19 for Lmx1b−−. *P < 0.05, **P < 0.01. (D) Female–male mounting percentage was not significantly different among Lmx1b+/−, Lmx1b+/+, and Lmx1b−/− littermates. (E) Female–male mounting latencies were not different. (F) Female–male mounting bouts were not different among estrous Lmx1b+/− and their female Lmx1b+/+ and Lmx1b−/− littermates. Both proceptive (G) and lordosis (H) quotients were not significantly different among test females. (I) The receptivity scores were not significantly different among the test females.

Fig. S7. Sexual preference of Tph2 knockout female mice. Data are from the same experiments as those in Fig. 3. (A) Tph2−/− females sniffed male targets later than their Tph2+/− littermates. Tph2+/− females sniffed male targets earlier than female targets. (B) Tph2−/− females sniffed male genital later than their Tph2+/− littermates. (C) Genital sniffing duration was analyzed in individual females as the duration of sniffing males minus the duration of sniffing females. (D) Latencies for sniffing the head were not different between Tph2+/− and Tph2−/− littermates. (E) Head-sniffing bouts were analyzed in individual females as the duration of sniffing males minus the duration of sniffing females. (F) A higher percentage of female–female mounting occurred in Tph2−/− females than in their Tph2+/− littermates.
Fig. S8. Bedding preference of female mice. \( n = 9 \) for \( Tph2^{+/+} \), \( n = 10 \) for \( Tph2^{+-} \), \( n = 9 \) for \( Tph2^{-/-} \). * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \). (A) More \( Tph2^{-/-} \) female mice than \( Tph2^{+/+} \) or \( Tph2^{+-} \) females stayed on female bedding longer than on male bedding. (B) \( Tph2^{-/-} \) female mice stayed on female bedding for longer duration than on male bedding. (C) Analysis of bedding preference in individual females: duration on male bedding minus duration on female bedding for the same mouse was calculated.
Fig. S9. Hormone concentrations in the plasma. (A and C) Diestrous females: $n = 6$ for $Tph2^{+/-}$; $n = 8$ for $Tph2^{-/-}$; $n = 8$ for $Tph2^{-/-}$. (B and D) Estrous females: $n = 6$ for $Tph2^{+/-}$; $n = 7$ for $Tph2^{-/-}$; $n = 7$ for $Tph2^{-/-}$. (A) Estradiol concentrations were not different among all indicated genotypes of diestrous females. (B) Estradiol concentrations were not different among all genotypes of estrous females. (C) Testosterone concentrations were not different among all genotypes of diestrous females. (D) Testosterone concentrations were not different among all genotypes of estrous females.
Fig. S10. Sexual preference of estradiol-treated ovariotomized (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−/−.