Development of the main olfactory system and main olfactory epithelium-dependent male mating behavior are altered in G_α-deficient mice

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Contributed by Lutz Birnbaumer, August 10, 2016 (sent for review May 5, 2016; reviewed by Cheil Moon and Daniel Storm)

In mammals, initial detection of olfactory stimuli is mediated by sensory neurons in the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). The heterotrimeric GTP-binding protein G_α is widely expressed in the MOE and VNO of mice. Early studies indicated that G_α expression in VNO sensory neurons is critical for directing social and sexual behaviors in female mice [Oboti L, et al. (2014) BMC Biol 12:31]. However, the physiological functions of G_α in the MOE have remained poorly defined. Here, we examined the role of G_α in the MOE using mice lacking the α subunit of G_α. Development of the olfactory bulb (OB) was perturbed in mutant mice as a result of reduced neurogenesis and increased cell death. The balance between cell types of OB interneurons was altered in mutant mice, with an increase in the number of tyrosine hydroxylase-positive interneurons at the expense of calbindin-positive interneurons. Sexual behavior toward female mice and preference for female urine odors by olfactory sensory neurons in the MOE were abolished in mutant male mice. Our data suggest that G_α signaling is essential for the structural and functional integrity of the MOE and for specification of OB interneurons, which in turn are required for the transmission of pheromone signals and the initiation of mating behavior with the opposite sex.

Pheromones evoke diverse social and sexual behaviors in animals of the same species. In mammals, initial detection of olfactory stimuli is mediated by sensory neurons in the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). Early studies indicated that the VNO is largely responsible for the detection of pheromones and the consequent direction of social and sexual behaviors (1). VNO sensory neurons (VSNs) express distinct types of vomeronasal receptors: type 1 (V1Rs) in the apical region and type 2 (V2Rs) in the basal region. These receptors couple, respectively, to G_α and G_βγ members of the heterotrimeric GTP-binding protein family. It has been suggested that, following ligand binding to V1Rs and V2Rs, the G_α subunits released from G_α and G_βγ activate phospholipase C and increase the intracellular concentration of Ca_2^+ ions. These Ca_2^+ ions activate inward rectifying K_+ (SK3) channels; subsequently activating Ca_2^+/activated Cl^- channels (CACCs).


Reviewers: C.M., Daegu Gyeongbuk Institute of Science and Technology; and D.S., University of Washington.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1613026113/-/DCSupplemental.

Significance

Early studies indicated that two olfactory systems in mammals play distinct roles: The main olfactory system detects general odorants, and the accessory olfactory system detects pheromones. Using heterotrimeric G_α protein-deficient mice, we show that pheromone detection by the main olfactory system is necessary for the initiation of mating behavior toward the opposite sex and that G_α plays a key role in this process by regulating the development of the main olfactory epithelium and the differentiation of dopaminergic interneurons in the olfactory bulb. Our findings provide the basis for identifying pheromone receptors in the main olfactory system and for understanding the hyposmia found in Parkinson’s disease, in which the number of inhibitory dopaminergic interneurons is increased in the olfactory bulb.


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and fail to thrive because of their inability to suckle. However, the rare $G_{\alpha ol}$ homozygotes that survive to sexual maturity are fertile and mate (10). Thus, it remains to be elucidated why AC3- and CNGA2-deficient mice, unlike $G_{\alpha ol}$ mutant mice, lose the preference for the opposite sex and which G proteins regulate this signaling pathway in OSNs of the MOE.

In OSNs, the expression of $G_{\alpha ol}$, a subunit of $G_{\alpha o}$, is largely absent from the olfactory bulb (OB), with the exception of neurons in the medial part that receive incoming axons from a small subpopulation of OSNs (11). Targeted deletion of $G_{\alpha ol}$ disrupts the aggressive behavior of males without affecting the preference for the opposite sex (12). By comparison, $G_{\alpha o}$, a subunit of $G_{\alpha o}$, is widely expressed in OSNs of the MOE (11). Attempts have been made to delete $G_{\alpha o}$ in the MOE using Cre recombinase expressed under the control of the olfactory marker protein (OMP) gene promoter. However, conditional knockout of $G_{\alpha o}$ (c$G_{\alpha o}$-knockout) using OMP-Cre results in the loss of $G_{\alpha ol}$ expression specifically in the VNO, apparently leaving expression in the MOE intact (13). Such a VNO-restricted deficiency of $G_{\alpha ol}$ disrupts only the aggressive behavior of males without affecting the preference for the opposite sex (13, 14). Collectively, these observations suggest that the MOE plays a role in detecting pheromones that trigger sexual behaviors such as preference for the opposite sex and initiation of sexual activity. They also raise the question of which G protein conveys pheromone signaling in the MOE.

We show here that male $G_{\alpha o}$-knockout ($G_{\alpha o}$-/-) mice, which lack $G_{\alpha o}$ in both the MOE and VNO, fail to develop a preference for female urine odors and are unable to engage in mounting behavior with estrous females. Although mutant mice exhibited a reduction in MOE mass, they retained an ability to detect general odors comparable to that of WT ($G_{\alpha o}$/+) mice. Surprisingly, the number of tyrosine hydroxylase (TH)-positive interneurons was increased in the OB of $G_{\alpha o}$-/- mice, whereas the number of calbindin (CB)-positive interneurons was reduced. Our data indicate that $G_{\alpha ol}$ plays important roles in the regulation of neurogenesis and cell-type specification in the main olfactory system and thus in the detection of pheromones. They also implicate a $G_{\alpha ol}$-mediated sensory contribution of the MOE to the preference for the opposite sex and mating initiation.

Results

$G_{\alpha ol}$ Ablation Leads to an Altered MOE. $G_{\alpha o}$-/- mice were obtained by crossing heterozygous $G_{\alpha o}$-/- mice as previously described (15). These mice have a low birth rate and rarely survive to adulthood because of severe neurological deficits, including hyperactivity and seizures. We compared the overall structure of the MOE and VNO in adult $G_{\alpha o}$-/- and $G_{\alpha o}$+/+ mice (Fig. 1 I and J). H&E staining of corresponding coronal sections of the adult mouse brain revealed that the sensory epithelia of the MOE and VNO were atrophied in $G_{\alpha o}$-/- mice compared with $G_{\alpha o}$+/+ mice (Fig. 1 A and B). Immunohistofluorescence analyses of $G_{\alpha o}$+/+ mice showed that OMP and neuron-specific β-III tubulin (Tubb3) are present throughout the VNO sensory epithelium (VNE), as previously reported (16). $G_{\alpha ol}$ protein is also present in VSNs as well as at the luminal surface containing the microvilli that extend from these neurons (Fig. 1 C and E). Such immunoreactivity was not detected in $G_{\alpha o}$-/- mice, validating the specificity of the immunohistological approach. The posterior accessory olfactory bulb (AOB), which contains incoming axons from the basal VNO, was also atrophied in $G_{\alpha o}$-/- mice (Fig. S1), consistent with previous observations that $G_{\alpha ol}$ ablation results in degeneration of the basal layer of the VNE and the posterior AOB (13, 17). In the MOE of adult $G_{\alpha o}$+/+ mice, $G_{\alpha ol}$ was detected in most cells along the apical–basal axis, including OMP+ mature OSNs, Tubb3+ immature OSNs, and sustentacular cells (Fig. 1 D and F). Because $G_{\alpha ol}$ protein is highly targeted to the plasma membrane, whereas the OMP protein is cytosolic, it was necessary to locate $G_{\alpha ol}$ and OMP expression precisely. Postnatal day 7 (P7) mice, in which the nasal bone was fully calcified and nose regions containing the MOE and VNO was cryosectioned without a decalcification process. In situ hybridization with a $G_{\alpha ol}$-antisense RNA probe revealed wide expression of $G_{\alpha ol}$ mRNA in the entire MOE along the apical–basal axis except in cells adjacent to the lamina propria, confirming the immunohistochemical results (Fig. S2B). Immunohistochemistry using an anti-OMP antibody revealed that the presence of OMP was limited to the apical region (Fig. S2C). Again, such $G_{\alpha ol}$ mRNA signals were not detected with $G_{\alpha ol}$-sense RNA probes, validating the specificity of our approach (Fig. S2, Upper). Importantly, $G_{\alpha ol}$ ablation reduced MOE thickness by ~13% and total cell number.
by ∼20% (Fig. 1 G and H). These results thus indicate that Gαo is essential for maintenance not only of the VNE, but also of the MOE.

**Gαo Ablation Impairs MOE Neurogenesis.** We next investigated how Gαo ablation resulted in MOE atrophy. TUNEL analyses revealed a 4.5-fold increase in the number of apoptotic cells in the MOE of adult Gnao−/− mice (Fig. 2 A and E). In addition, the number of proliferating cell nuclear antigen (PCNA)-positive proliferating cells, mammalian achete-scute homolog 1 (Mash1)-positive progenitor cells, and sex determining region Y-box 2 (Sox2)-positive basal progenitor cells in the MOE were significantly reduced in Gnao−/− mice (Fig. 2 D and F). The number of Sox2+ apical sustentacular cells, all of which originate from a common progenitor (18), was also reduced in the MOE of Gnao−/− mice (Fig. 2 D and F). These results thus indicate that the overall reduction in the size of the MOE induced by Gαo ablation was the result of the combined effects of reduced neurogenesis and increased cell death. Consistent with these findings, the olfactory nerve layer (ONL) of the OB, which comprises projecting axons of OMP+, neural cell adhesion molecule (NCAM)-positive and Gαo+ OSNs, was thinner in the mutant (Fig. S3). In contrast, microtubule-associated protein 2 (MAP2) staining in the glomerular layer (GL), where dendrites of mitral cells make synaptic contact with incoming OSN axons, and in the external plexiform layer (EPL) of the OB appeared unaffected in Gnao−/− mice. These observations suggest that the structural changes of the MOE induced by the loss of Gαo may not extend beyond the GL.

**Gαo Ablation Alters Cell-Type Specification in the OB.** Neurotransmission between pre- and postsynaptic fibers in the GL of the OB is extensively modulated by periglomerular interneurons that exclusively express tyrosine TH, CB, or calretinin (CR) (19). Deprivation of presynaptic input from the ONL in rodents, either by treatment with zinc sulfate or by naris occlusion, was previously shown to reduce both TH expression and dopamine levels in the GL of the OB (20, 21), whereas naris reopening resulted in the generation of new TH+ interneurons (22). We therefore expected that the reduced thickness of the ONL in the OB of Gnao−/− mice would be associated with a reduction in TH expression or a reduced number of TH+ periglomerular cells. However, we found that the number of TH+ interneurons was actually increased ∼1.9-fold in the GL and ∼2.5-fold in the EPL of Gnao−/− mice (Fig. 3 A–C, F, and G). One possible explanation for these results is that OB progenitor cells might tend to differentiate toward TH+ interneurons in the absence of Gαo, in which case the number of CB+ interneurons, which differentiate from the same progenitor cells. Indeed, we found that the number of CB+ interneurons in the GL was reduced by ∼13% (Fig. 3 D and H). In contrast, the number of CR+ interneurons, which arise from a different population of progenitor cells, was not affected by Gαo ablation (Fig. 3 E and I).

Although the receptors for various neurotransmitters, including glutamate, serotonin, opioids, acetylcholine, and ATP, are thought to be coupled to Gi or Gαo, the dopamine type 2 receptor (D2R) has been unambiguously shown to signal mostly, if not exclusively, via Gαo (23). In rodents, the embryonic OB contains immature GABA+ progenitor cells that subsequently differentiate into mature TH+ cells (24). We next tested whether the suppression of D2R-activated Gαo signaling mimicked the effect of Gαo ablation on the number of TH+ cells generated by OB progenitors. To this end, we isolated OB neurons from WT mice at embryonic day 16.5 (E16.5) and cultured the cells for 1 d in the presence of the D2R antagonist sulpiride to block Gαo signaling. Addition of sulpiride did not affect the number of TH+ neurons, and pretreatment with it did not affect the dopamine-induced increase in the number of TH+ cells: treatment with the D2R agonist ropinirole was similarly without effect (Fig. S4). These results suggest that OB progenitor cells at E16.5 are already predisposed to become either TH+ or CB+ neurons in a cell-autonomous manner and that the prospective TH+ or CB+ neurons are not converted to other types of interneurons in response to D2R signaling.

To examine whether the neurochemical phenotypes of OB interneurons are specified at E16.5 by Gαo, we analyzed dissociated neuronal cultures prepared from the OB of E16.5 Gnao−/− mice or their Gnao+/+ littermates. Consistent with in vivo results, the
number of TH⁺ neurons in Gnao−/− cultures was increased ~2.2-fold compared with Gnao+/+ cultures (Fig. 4 A and C), whereas the number of CB⁺ neurons was reduced by approximately one-half (Fig. 4 B and D). These data indicate that more embryonic OB progenitor cells are predisposed to differentiate into TH⁺ interneurons than into CB⁺ interneurons in the absence of Gnao, and that Gnao therefore may play a pivotal role in regulating the specification of TH⁺ and CB⁺ interneurons in the OB.

Gnao Ablation Impairs Sexual Behavior. We next tested whether Gnao ablation in the MOE and VNO impairs olfactory sensation, as measured by the hidden food detection test. The latency to finding the hidden food and the total time spent in the vicinity of the food did not differ in Gnao−/− and Gnao+/+ male mice (Fig. 5 A and B). Thus, the overall function of odor detection was not affected in Gnao−/− mice, despite the evident atrophy of the MOE and VNO in these animals. These results suggest that the contribution of Gnao to food detection is minimal.

We then examined whether Gnao ablation affects pheromone detection. Gnao−/− mice are born in reduced number, have a high death rate, and manifest increased locomotor activity (15). We therefore designed a noninvasive experiment to test whether male mice are able to smell pheromones in female urine. In this experimental paradigm, soiled bedding from males, estrous females, or anestrous females was randomly placed in three alternate compartments of a five-compartment apparatus, with fresh bedding placed in the two intervening compartments. Gnao−/− male mice spent more time in the bedding soiled by estrous females than in the other bedding types and spent the least amount of time in the fresh bedding. In contrast, Gnao−/− male mice spent similar amounts of time in all types of bedding, including fresh bedding, thus exhibiting no preference for any compartment (Fig. 5 C). These results suggest that Gnao−/− male mice have lost the ability to detect pheromones in female urine, even though their ability to detect food odors remains intact.

To examine mating behavior, we introduced an unfamiliar estrous WT female mouse that was highly sexually receptive into the home cage of a male mouse. Gnao−/− male mice intensively investigated the anogenital area of the female mouse and attempted to mount the female, but Gnao−/− males showed little interest in the female, exhibiting a greatly reduced frequency of anogenital investigation and an almost complete absence of mounting behavior (Fig. 5 D). Gnao−/− male mice showed no gross anatomic defects in reproductive organs, including seminal vesicles, testes, and epididymis (Fig. S5), suggesting that testosterone levels are similar in these mutant mice and Gnao−/− males. The lack of anogenital investigation by Gnao−/− male mice therefore is not likely attributable to a secondary effect of defective reproductive organs or reduced testosterone levels but rather reflects impaired preference for the opposite sex. Taken together, these results suggest that Gnao−/− male mice do not show a sexual partner preference because they lack the ability to detect pheromone cues.

Discussion

Mouse genetic studies have suggested that the MOE-OB and VNO-AOB pathways are essential mediators of pheromone-triggered social and sexual behaviors. Deletion of VNO-specific genes such as Trpc2 has been found to disrupt gender identification while sparing sex preference (3, 4). By comparison, both preference for the opposite sex and the initiation of sexual behaviors, such as anogenital investigation and mounting, are lost as a result of deletion of the MOE-specific genes Adcy3 or Cnga2 (8, 9). Global deletion of the gene encoding Gna2 (Gnai2), which is expressed in the apical VNO and anterior AOB (12), or conditional Gnao knockout (cGnao−/−) using an OMP-Cre construct disrupts innate and maternal aggressive behaviors without affecting sexual behavior (13, 14). We show here that Gnao−/− male mice barely investigated the anogenital area of female mice and did not show a preference for bedding soiled by estrous females. They therefore lacked the preference for a female sex partner and largely failed to engage in sexual behavior. Taken together with previous mouse genetic studies, our results suggest that, although both the MOE and VNO detect pheromone signals and trigger mating behavior, the MOE appears to make a greater contribution to the preference for the opposite sex and initiation of sexual behavior, whereas the VNO mediates gender identification (Fig. 6).

Structural alterations were widespread along the apical–basal axis in the MOE of Gnao−/− mice, affecting OMP⁺ OSNs as well as progenitor cells in the basal region and sustentacular cells in the apical region. Nevertheless, food-detection ability appeared normal in the mutant animals, with only pheromone detection being selectively impaired (Fig. 5). General odors, such as those of food, are detected by canonical OMP⁺ OSNs in the MOE, and their intracellular signals are transmitted via the α subunit of the G protein Gαolf and the cAMP signaling cascade (25, 26). Therefore, intact Gαolf accounts for the normal ability of Gnao−/− mice to detect food pellets (Fig. 5 A and B) but cannot
replace the function of $G_o$ in transducing pheromone signals in the MOE. In the early postnatal period between P4 and P7, the expression of $G_{oa}$ protein or Gnao mRNA is widely detected in the MOE, whereas OMP expression, the hallmark of mature OSNs, is limited to the apical region (Figs. S2 and S6). OMP expression is the hallmark of mature OSNs, which extend dendritic knobs to the ciliary layer (27). $G_{ot}$, was detected in a subset of dendritic knobs in P4 and in adult MOE. Further studies are necessary to elucidate whether the $G_{ot}$, dendritic knobs at the ciliary layer functions in pheromone transduction.

Previous studies using an independently generated $G_{oa}$-deficient mouse model also reported that homozygous-null animals retained the ability to locate food but had lost the specific sense of Nasonov pheromones such as geraniol and citralva (28). Animals in a state of food deprivation will work much harder to obtain food in an operant task than animals fed ad libitum, because hunger modulates the sensitivity to sensory cues associated with food and influences behavioral responses (29). Thus, it is possible that the $G_{oa}$-driven ability to detect food may be enhanced after food deprivation in $G_{oa}$-deficient mice. The fact that WT and $G_{oa}$-deficient mice have a similar ability to locate food may indicate that the neural circuits of hypothalamic neurons that sense energy deficits and induce food seeking and consumption may be functional in the brain-wide absence of $G_{oa}$. Thus, it is likely that the lack of preference for the opposite sex in Gnao−/− males is attributable to the selective failure of pheromone detection. However, we cannot rule out the possibility that neural circuits in other areas that control the initiation of sexual behavior toward the opposite sex are selectively damaged in the absence of $G_{oa}$.

Our results support a recent study showing that $G_{oa}$ and $G_o$, mediate alternate signaling pathways in an individual OR-expressing OSN in a ligand-selective manner. Applying RNA-sequencing technology to single OSNs, Scholz and colleagues found that $G_{oa}$ and $G_o$, are coexpressed in the same neuron, where they independently activate distinct signaling pathways in a stimulus-dependent manner upon the binding of diverse ligands to the same Olfr73 olfactory receptor (30): $G_{oa}$, activation leads to an efflux of $\text{Ca}^{2+}$ that is independent of canonical pathways, including activation of AC3, increases in $\text{Ca}^{2+}$ stemming from extra- or intracellular sources, or phosphatidylinositol 3-kinase–dependent signaling (31); and, as mentioned in the Introduction, $G_o$,-derived GIRQ activates GIRK channels independently of TRPC2. Thus, in the VNO, unlike other sensory organs, SK3, GIRK, and CACCC channel activation of its VSNs leads to $K^+$ influx and $\text{Ca}^{2+}$ efflux (2). AC3−/− and Gnao−/− mice exhibit similar phenotypes (Fig. 6), and the MOE expresses multiple types of adenylyl cyclases (ACs) including AC2, AC3, and AC4 (32). It would be of interest to elucidate whether $G_o$, activates any of these ACs. Several studies have reported that specialized OSNs express trace amine-associated receptors (33), guanylyl cyclase-D (34), or TRPC2 (35). $G_o$, is found in TRPC2-expressing OSNs in the MOE, some of which do not express AC3 or $G_{oa}$. It is possible that $G_o$, may regulate pheromone signals in these specialized neurons of the MOE.

Interestingly, most $G_{oa}$,-expressing neurons in the basal MOE were devoid of OMP expression in the P4–7 MOE, whereas most $G_o$-expressing neurons expressed OMP in the P7 VNE (Fig. S2). Such OMP expression in a subpopulation of $G_{oa}$, cells in the apical MOE during early postnatal periods might cause insufficient deletion of Gnao by OMP-driven Cre recombinase in the MOE of OMP-Cre;Gnao−/− mice, resulting in a Gnao1/− phenotype in the MOE of Gnao−/− mice, as previously surmised (13). Indeed, Gnao−/− mice have been shown to retain WT male sexual preference and mating behavior (14), as summarized in Fig. 6.

Ablation of $G_{oa}$ reduced the thickness of the MOE and VNO (Fig. 1). In agreement with a previous report showing increased cell death in the VNO in Gnao−/− mice (17), we found that TUNEL− apoptotic cells were increased in the MOE of Gnao−/− mice (Fig. 2), indicating that $G_{oa}$ is critical for neuronal cell survival in both the MOE and VNO. We also found that the numbers of Mash1− and Sox2− basal cells, as well as PCNA proliferating cells, were reduced in the MOE of Gnao−/− mice. Taken together, these data indicate that $G_{oa}$ signals are necessary not only for the survival of OSNs but also for the neurogenesis of MOE neurons.

TH+ dopaminergic periglomerular interneurons inhibit synaptic transmission between OSNs and mitral cells in the OB by blocking dopamine and GABA. It has been shown that the number of periglomerular TH+ neurons is markedly increased in pathological conditions such as Alzheimer’s disease and Parkinson’s disease (36). Olfactory dysfunction is considered a reliable marker of these diseases that precedes the typical clinical manifestations by many years (37); however, the mechanisms underlying this association are poorly understood. We have now shown that $G_{oa}$, ablation impairs pheromone detection and increases the number of TH+ OB interneurons. The increase in the number of TH+ interneurons in the OB likely does not arise from simple activation of the TH gene promoter but rather is the result of cell-type switching of neurochemically immature prospective OB interneurons triggered by the loss of $G_{oa}$.

The increased dopaminergic tone in the OB of Gnao−/− mice may reflect a compensatory mechanism triggered by the early defective development of the MOE, which likely interferes with pheromone signal transduction at the level of the GL. Consistent with our in vivo results, the ratio of TH+ neurons to Tubb3+ neurons was also increased in dissociated cell cultures prepared from the OB of E16.5 Gnao−/− mice, whereas the ratio of CB+ neurons to Tubb3+ neurons was decreased. Given that TH+ and CB+ neurons originate from a common progenitor pool in the dorsal lateral ganglionic eminence during the embryonic period and CR+ neurons are derived from progenitor cells located elsewhere (38), it is likely that the reciprocal changes in the number of TH+ and CB+ cells in the OB of Gnao−/− mice are attributable to altered cell-type specification rather than to
the induction of TH expression or enhanced survival of TH⁺ neurons. Further studies are necessary to elucidate how Gα regulates the expression of intrinsic transcription factors that specify the phenotype of these various interneurons.

Although the binding of a variety of neurotransmitters to cognate receptors is thought to trigger Gα signaling, the functions of Gαo in the brain remain largely unknown, partly because of the functional and severe neurological phenotypes of Gnao−/− mice (15). We now provide evidence that Gαo regulates adult neurogenesis and neuronal survival in the MOE and contributes to cell-type specification of OB interneurons. Our results may provide a basis for the identification of pheromone receptors in the MOE.

Materials and Methods

Standard procedures and methods were used for histological analysis, immunohistochemistry, in situ hybridization, TUNEL staining, and statistical analyses. P < 0.05 was considered statistically significant. Expanded methods and materials are available in SI Materials and Methods.

Mice. Gnao−/− and Gnao+/− littermate mice were obtained by interbreeding Gnao−/− littermates. Adult male mice were used for all experiments and analyses unless indicated otherwise. All experimental procedures were reviewed and approved by the Institutional Animal Research Ethics Committee of Ajou University and by the National Institute of Environmental Health Sciences.

19. Brotchie ML, et al. (2000) The male sexual behavior test was performed in a clean cage into which a male mouse was placed. Estrous female mice were prepared as previously described (39), and the sexual behavior of the male was recorded for 1 h under red-light illumination.
20. Zhang M, et al. (2012) Use of an estrous female mouse to locate the food-containing box and the total time spent near the food was determined. Each mouse was subjected to three trials.
21. Male Sexual Behavior Test. The male sexual behavior test was performed in a clear, closed acrylic chamber with a black box attached on each side. Food pellets were hidden in only one of the black boxes. The mouse was deprived of food for 1 d and was placed at the midline of the chamber. Behavior was recorded for 10 min under red-light illumination, and the time required for the mouse to locate the food-containing box and the total time spent near the food was determined. Each mouse was subjected to three trials.