SUPPLEMENTARY MATERIALS

Animals

Animals were housed under a reversed 12 hr : 12 hr light-dark cycle with the light cycle starting at 1 AM. All animals were bred and tested in compliance with the University of California San Francisco Institutional Animal Care and Use Committee and Live Animal Resource Center. The Cnga2 mutant strain is maintained on a mixed background of C57Bl/6J and 129SvEv. To obtain hemizygous males, heterozygous females (kindly provided by J. Ngai) were mated to WT C57Bl/6J or 129SvEv males. The resulting litters were trimmed to 4 - 5 pups 6 - 10 hours after birth such that all mutant males and at least one WT male or female littermate remained. Male mutants were identified by the virtual absence of a milk spot in the abdomen. The dams were given peanut butter (Skippy squeezable) and high-caloric food pellets (Labdiet 5013) to allow the mutant pups to thrive. Pups were weaned 3 - 4 weeks after birth. Genotypes were determined by PCR of tail DNA using the following primer pairs: 5’-TGGCTGGTCTGGATTACTTCTCAG-3’ and 5’-CGCAATTCTTGGGTCTTTGACC-3’ to amplify the WT band; 5’-GCTATTGGCTATGACTGGCACAACAG-3’ and 5’-TGGATACTTCTCGGCAGGAGCAGGTTG3’ to amplify the neomycin present in the targeting vector. Upon weaning, WT and mutant males were group housed by sex, and fresh peanut butter and pre-wetted food pellets were provided every 2 - 3 days for 10 days. The mice were subsequently given dry pellets on the cage floor for 5 days, after which the food pellets were only provided using the regular overhead feeders. At 7 - 8 weeks after birth, the males were moved to individual housing. Adult mutants (26.4 ± 1.1 g; mean ± s.e.m.; n = 7) could not be distinguished reliably from WT male siblings (30.3 ± 1.7 g; n = 4; P = 0.07) by size. Behavioral testing commenced 7 - 10 days after the animals had been singly housed. Serum testosterone was measured using an EIA kit (DRG International) from adult sibling WT and Cnga2 mutant males.
This test revealed a testosterone concentration of 0.9 ± 0.3 nM in WT (n = 8) and 1.7 ± 0.5 nM in mutant males (n = 12; P = 0.5).

**RT-PCR analysis of Cnga2 expression**

VNO and MOE were carefully dissected from 3 WT males. Total RNA was prepared with Trizol reagent (Invitrogen) and 1 µg from each tissue was subjected to DNaseI treatment (Amplification Grade DNaseI, Invitrogen). Half of this RNA was then reverse transcribed into cDNA with oligo dT and the SuperScript RT III system (Invitrogen) while the other half was subjected to the same reaction lacking RT (RT– control). 0.5 µL of the RT (or RT– control) was subjected to PCR using Taq polymerase (Qiagen) and primers to detect Cnga2 (5’-GAACAAGGGCTCCTGGTCAAAGAC-3’ and 5’-GTAAAGGCAGTAAATGTACTCCCTAG -3’) and Omp (5’-GAGAAGAAGCAGGATGTTGAGAAGC-3’ and 5’-CGTCTGCCTCATTCCAATCCATGG-3’) in the same reaction. With these primers PCR products of ~ 400 bp and ~ 250 bp are expected for Cnga2 and Omp, respectively. We performed PCR for 20, 25, 30 and 35 cycles for cDNA prepared from both tissues and observed that the PCR appeared to saturate at 30 cycles. The RT-PCR was done on total RNA prepared from 2 independent dissections. The PCR products from the MOE and the VNO were subcloned (Topo II, Invitrogen) and sequenced to confirm that we had amplified Cnga2 and Omp.

**Behavioral Analysis**

Animals were 8 - 20 weeks old when used for testing. All behaviors were assayed between 1.5 - 8 hours after lights were switched off. The behavioral tests were recorded on MiniDV tapes with a Sony DCR-PC330 Handy-Cam using the inbuilt infrared illumination. All behavioral parameters were scored subsequently with Noldus Observer 5.0.
*Mating assay*

Wildtype females (C57Bl/6J or 129SvEv, n = 30) determined to be in estrus by visual examination of their genitalia were used as stimulus females in the mating tests. An estrus female was introduced into the singly housed male’s homecage and the interactions were recorded for 30 minutes. Males were never tested > 2 times a week and a minimum of 72 hours elapsed between each mating test. Prior to the first mating test males had not been exposed to females since weaning. All males were tested 2 - 3 times, using different females in each assay. The assays were scored for chemoinvestigation of anogenital area, grooming, mounting, intromission, ejaculation, and aggression. While chemoinvestigation probably involves sniffing and licking, we use sniffing, chemoinvestigation, and chemosensory exploration interchangeably throughout the text to denote investigation of the anogenital region of a conspecific in mating and aggression, and of the urine wetted swab in preference assays.

To determine whether mutants would mate in a longer assay we cohoused females with resident mutant and WT males (n = 5, each) and checked for vaginal plugs (indicative of ejaculation) daily for ~ 10 days. All females housed with WT males plugged at least once and produced litters whereas only one female cohabiting with mutants plugged once and gave birth.

*Resident-intruder aggression test*

All residents were singly housed and had previously been tested in the mating assay. The intruder was an 8 - 10 week old, gonadally intact 129SvEv male, group housed since weaning. The intruder was placed in the home cage of the resident and the interactions were recorded for 15 minutes. Resident males were tested for aggression in 2 - 3 assays such that each animal was tested only once within 48 hours and never > 2 times a week. The assays were scored for sniffing,
grooming, attacking, chasing, mounting, and tail rattles displayed by either the resident or the intruder. An episode of attack includes one or more instances of biting, tumbling, or wrestling.

When attacked by a WT intruder the mutant fled or defended himself, suggesting that even under attack mutants are unlikely to initiate fights. Sexually experienced males are more aggressive than naïve males. However, individual housing induces aggression in males independent of mating experience. Our male residents are singly housed for several weeks, making it unlikely that the reduced aggressivity in Cnga2<sup>−/−</sup> results solely from mating deficits.

**Urine preference test**
Singly housed WT and Cnga2 mutant males not previously tested for either mating or aggression were used to determine whether they exhibited a preference for male or female urine. On the day of testing, urine was collected from WT males or females by gently gripping the animal by the scruff of the neck and holding the animal over a fresh piece of Parafilm. In this position most animals yielded 50-300 µL of urine. Urine was pooled from several animals of the same sex and kept on ice until the time of the assay. A fresh, sterile, 1” × 1” cotton pad, handled only with gloves, was wetted with 50 µL of male urine, female urine or saline. Two swabs wetted with different odorant sources were presented simultaneously to the resident at random locations in the homecage for 5 minutes. The frequency and duration of sniffing or non-sniff interactions (NSI) of each swab were recorded. NSI includes carrying around the swab, tearing it up with mouth and forepaws, or pushing it around. In summary, the mutants appear to interact with the swabs in a manner similar to WT males but they do not chemoinvestigate conspecific odors, thereby possibly resulting in the observed lack of preference for female urine over male urine or saline (not shown).
Data Analysis

Behavioral parameters were scored using Observer 5.0 (Noldus). We designated particular keystrokes to represent different behavioral parameters. For mating, these parameters included male mounting female, intromission by male, ejaculation, female mounting of male (never observed), aggressive interactions, female rejection of male mounting attempts (kicking, assuming upright defensive posture), chemoinvestigation by male or female and grooming by male or female. For aggression, the parameters included mounting, tail rattling, chemoinvestigation, grooming, attacks and chases; these parameters were scored for both resident and intruder via different keystrokes. The behaviors were observed by playing back the video recordings on iMovie (G4 Macintosh) and keystrokes were entered as particular behaviors occurred. The Observer software automatically provides frequency, duration and latency for each behavioral parameter. These behavior logs were compiled and exported into Microsoft Office Excel and Matlab 7.0 (MathWorks) for data analysis and statistical testing. For animals tested multiple times (mating or aggression) each behavioral parameter was averaged for the animal across multiple trials. These numbers were averaged across the genotype for each behavioral parameter, and then imported into Matlab for statistical analysis. Since many of the variables did not exhibit a Gaussian distribution we utilized non-parametric tests of statistical significance to determine whether WT and Cnga2 mutants differed for various parameters of each behavioral subroutine. All $P$ values given in the text were determined using the Wilcoxon rank sum test. All statistically significant results presented in the text ($P < 0.05$) were also determined to be statistically significant using an additional non-parametric test, the Kolmogorov-Smirnov test ($ks$-test). The data presented in the study were scored by an observer (V.S.M.) not blinded with respect to genotype. Similar results were obtained when 50% of the assays were scored by an observer (N.M.S.) blind to the genotype and not involved in the direct setup and taping of the behavioral assays.