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Deficits in sexual and aggressive behaviors in *Cnga2* mutant mice

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Odors detected by the vomeronasal organ or the main olfactory epithelium (MOE) trigger social behaviors in many animals. It is unknown whether MOE neurons detect cues that initiate mating or aggression. We demonstrate that mice lacking functional CNGA2 (cyclic nucleotide–gated channel α2), which is required for odor-evoked MOE signaling, fail to mate or fight, suggesting a broad and essential role for the MOE in regulating these behaviors.

Activation of the MOE or the vomeronasal organ (VNO) by specific odors can trigger appropriate social behaviors in rodents and many other vertebrates. The MOE is thought to be involved in detecting cues that initiate nursing and suckling[1,2]. The VNO is required for aggression in mice. Targeted deletion of transient receptor potential cation channel c2 (*Trpc2*), a gene expressed in the VNO, attenuates the responsivity of VNO neurons to mouse urine and abolishes aggression[3,4]. The chemosensory regulation of mating may be more complex. Olfactory bulbectomy, which eliminates transmission of MOE and VNO signals, abolishes sexual behavior[2]. $Trpc2^{-/-}$ males, however, mate normally with females[3,4]. Taken together with additional studies[2,5], these experiments suggest that the MOE and the VNO may mediate mating in a redundant fashion. Alternatively, mating and aggression may be segregated such that the MOE regulates mating, whereas the VNO initiates fighting. To distinguish between these models, we examined mice deficient in odor-evoked activity in the MOE. Although there may be redundancy in the early steps of odor-evoked signaling[1,6], CNGA2 (also referred to as CNGα2 or OCNC1) is the only cyclic nucleotide–binding subunit of the CNG channel expressed in most MOE neurons[7]. CNGA2 is essential for odor-evoked activity in the vast majority of MOE neurons[8]. Because CNGA2 is not expressed in vomeronasal neurons[7] (**Fig. 1a**), mice lacking the *Cnga2* gene permit analysis of the MOE's contribution to mating independent of the VNO.

We examined male mating in $Cnga2^{-/Y}$ mice (*Cnga2* is X-linked) and their wild-type siblings. Male mating consists of several sequential subroutines, including chemoinvestigation, mounting and intromission[3]. We observed a striking deficit in each of these components of sexual behavior in $Cnga2^{-/Y}$ mice compared to wild-type males (**Fig. 1b,c**). The latency to first sniff the female was 64-fold longer in $Cnga2^{-/Y}$ mice (mean ± s.e.m.: 419.4 ± 90.0 s, $n = 11$) compared to wild-type male mice (6.5 ± 1.4 s, $n = 9$, $P = 6.0 \times 10^{-4}$). All wild-type males (9 of 9) mounted in these assays, whereas none of the mutants (0 of 11) were observed to mount. Such deficits in sexual behavior persisted in a mating assay lasting several days (**Supplementary Note** online). These data demonstrate a profound reduction in many components of sexual behavior in *Cnga2* mutants.

Male mice chemoinvestigate males as well as females[9]. We asked whether the sniffing deficit exhibited by $Cnga2^{-/Y}$ mice toward females would also extend to male intruders. Wild-type resident males typically sniff and attack intruder males[9]. $Cnga2^{-/Y}$ residents showed a substantial reduction in sniffing and aggression compared to the wild-type mice ($Cnga2^{-/Y}$: 5 of 11 sniffed and 1 of 11 attacked; wild-type: 9 of 9 sniffed and 6 of 9 attacked; **Fig. 2**). The latency to first sniff the intruder was 32-fold longer for $Cnga2^{-/Y}$ mice (mean ± s.e.m.: 364.0 ± 165.8 s, $n = 11$) compared to wild-type male mice (11.4 ± 3.0 s, $n = 9$, $P = 1.0 \times 10^{-3}$). Together with the reduction in chemoinvestigation of females, these results suggest that *Cnga2* mutant males show a deficit in sniffing conspecifics during mating and aggression. Unlike $Trpc2^{-/-}$ males[3,4], $Cnga2^{-/Y}$ mice did not mount intruder males. The mounting of male intruders by $Trpc2^{-/-}$ residents has led to a model suggesting that in the absence of fighting, male mice revert to a 'default' mode of mating indiscriminately with all conspecifics[4]. Our data provide a dissociation

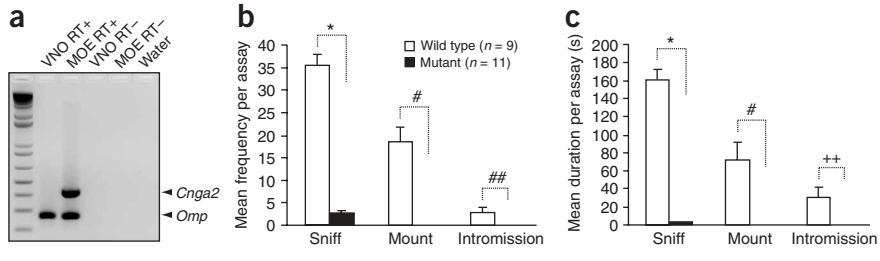

Figure 1 Loss of sexual behavior in $Cnga2^{-/Y}$ mice. (**a**) Reverse transcriptase–polymerase chain reaction (RT-PCR) experiments reveal expression of olfactory marker protein (*Omp*) and *Cnga2* in the MOE but only of *Omp* in the VNO. Shown is a typical gel run from an RT-PCR (35 cycles). The lowest band of the ladder is 200 bp with ~100 bp increments up to 600 bp. RT+, reaction with reverse transcriptase; RT–, reaction without reverse transcriptase; water, reaction without cDNA. Resident males were exposed to a female for 30 min. (**b**,**c**) The frequency (**b**) and duration (**c**) of mating subroutines are significantly diminished in mutants compared to wild-type mice. Animal care and handling was done in accordance with Institutional Animal Care and Use Committee guidelines. $^{*}P$, 2.0×10^{-4}; $^{\#}P$, 4.6×10^{-5}; $^{\#\#}P$, 7.0×10^{-4}; ^{++}P, 2.3×10^{-3}. Frequency represents the number of times a particular behavioral subroutine was observed in the assay. Error bars represent s.e.m.

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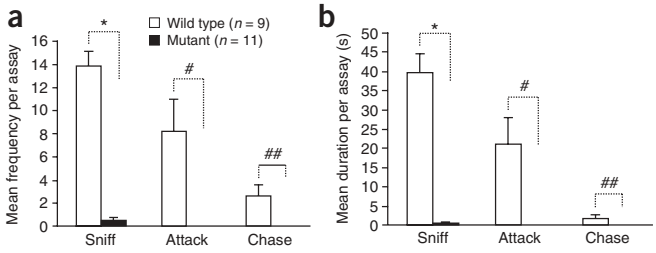

Figure 2 Loss of aggressive behavior in *Cnga2*$^{-/Y}$ mice. Resident males were exposed to a wild-type intruder male for 15 min. (**a**,**b**) The frequency (**a**) and duration (**b**) of sniffs, attacks and chases directed toward the intruder were significantly reduced in mutants compared to wild-type mice. **P*, 2.0×10^{-4}; #*P*, 5.7×10^{-3}; ##*P*, 2.3×10^{-3}. Frequency represents the number of times a particular behavioral subroutine was observed in the assay. Error bars represent s.e.m.

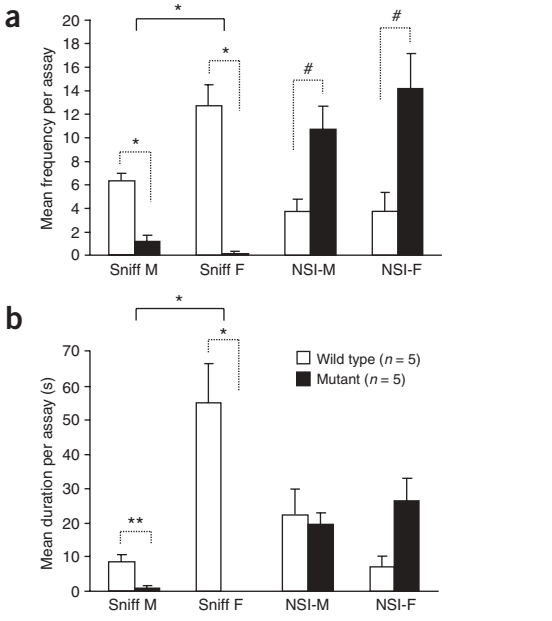

Figure 3 Loss of preference for female urine odors in *Cnga2*$^{-/Y}$ mice. Resident mutant and wild-type males were exposed to two cotton pads wetted with male (M) or female (F) urine for 5 min. (**a**,**b**) Wild-type mice sniffed female urine more frequently (**a**) and for longer duration (**b**) than did the mutants. *Cnga2*$^{-/Y}$ mice displayed more non-sniff interactions (NSI) with urine-wetted pads compared to the wild-type mice, although the total duration of NSI was similar between the two genotypes. **P*, 7.9×10^{-3}; ***P*, 1.6×10^{-2}; #*P*, 3.2×10^{-2}. NSI-F and NSI-M, NSI with female urine- and male urine-wetted swabs, respectively. Frequency represents the number of times a particular behavioral subroutine was observed in the assay. Error bars represent s.e.m.

between sexual behavior and the absence of aggression, suggesting that mating may involve a sensory contribution from the MOE.

The deficits in *Cnga2* mutants could result from a general avoidance of conspecifics. Alternatively, *Cnga2*$^{-/Y}$ mice may be unable to recognize conspecific odors that initiate social behaviors. *Cnga2* mutants groomed males and females in a manner similar to that of the wild-type residents, suggesting that these mutants did not avoid conspecifics entirely (**Supplementary Fig. 1** online). Moreover, we found that *Cnga2*$^{-/Y}$ mice fail to sniff male or female odors even when these were presented on a neutral substrate. We provided socially naïve wild-type and mutant males with female and male urine simultaneously on separate cotton pads. *Cnga2*$^{-/Y}$ mice sniffed these odors significantly less than did the wild-type mice (**Fig. 3a**). As expected from previous studies[10], wild-type males showed a preference for female urine. By comparison, *Cnga2*$^{-/Y}$ mice failed to sniff either pad preferentially. In addition to sniffing, wild-type and mutant males interacted with the pads by carrying them in their mouths, tearing them and pushing them around. Although *Cnga2*$^{-/Y}$ mice engaged in such non-sniff interactions with greater frequency than wild-type mice, the total duration of these interactions was similar between the two genotypes (**Fig. 3b**). Taken together, these results suggest that the mating and aggression deficits in *Cnga2*$^{-/Y}$ mice are unlikely to result solely from an avoidance of conspecifics or their odor cues.

Our data suggest an essential role for the MOE in mating and aggression. One explanation for the dual requirement for the MOE and the VNO in aggression is that these epithelia function in a parallel fashion to regulate fighting. In another model, attractant volatiles from conspecifics detected by the MOE may provoke chemoinvestigation involving physical contact, thereby permitting the VNO to access odorant cues. Diminished sniffing in *Cnga2* mutants may prevent the VNO from processing cues that initiate aggression. In support of such a sequential model of activation of first the MOE and then the VNO, conspecific volatiles activate synaptic target neurons of the MOE, whereas physical contact and chemoinvestigation seems essential for the activation of synaptic target neurons of the VNO[11,12]. Finally, the VNO may also detect volatile odors[2,13]. Thus, the reduced sniffing by *Cnga2*$^{-/Y}$ mice may prevent the VNO from gaining access to aggression-modulating volatile cues. It will be interesting to determine the mechanisms underlying the diminished sniffing in these mutants. In any event, an intact VNO is not required for mating[3,4], suggesting that the MOE also processes cues that regulate sexual behavior. In future studies, it will be important to determine whether prior social experience bypasses the requirement for a functional MOE in chemoinvestigation, mating and aggression. Finally, some MOE neurons do not express CNGA2 and use a distinct odor-evoked signaling pathway[2]. Our data suggest that this subpopulation cannot initiate mating or fighting in the absence of MOE neurons expressing functional CNGA2.

We cannot exclude the possibility that central deficits, including aberrant connectivity, produce the behavioral phenotypes we observe in *Cnga2*$^{-/Y}$ mice. CNGA2 is also expressed in several brain regions[14]. Nevertheless, *Cnga2*$^{-/Y}$ mice resemble wild-type mice in many behavioral tests, including grooming (**Supplementary Fig. 1**) and operant conditioning[2]. Finally, *Cnga2*$^{-/Y}$ mice have circulating levels of testosterone that are similar to those in wild-type mice (**Supplementary Note**), suggesting that the behavioral phenotypes are unlikely to arise from testosterone deficits in adults. The behavioral deficits observed in *Cnga2*$^{-/Y}$ mice resemble the phenotypes resulting from adult bulbectomy[15]. These surgically lesioned males fail to chemoinvestigate conspecifics and do not mate or fight. Our results are therefore consistent with a role for CNGA2-expressing MOE neurons in regulating chemoinvestigation, mating and aggression.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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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 C57Bl6/J and 129SvEv. To obtain hemizygous males, heterozygous females (kindly provided by J. Ngai) were mated to WT C57Bl6/J 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'-TGGCTGGTGCTGGATTACTTCTCAG-3' and 5'-CGCAATTTCTTGGGGTCTTTGACC-3' to amplify the WT band; 5'-GCTATTCGGCTATGACTGGGCACAACAG-3' and 5'-TGGATACTTCTCGGCAGGAGCAAGGTG-3' 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'-GAGAAGAAGCAGGATGGTGAGAAGC-3' and 5'-CGTCTGCCT CATTCCAATCCATGG-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.

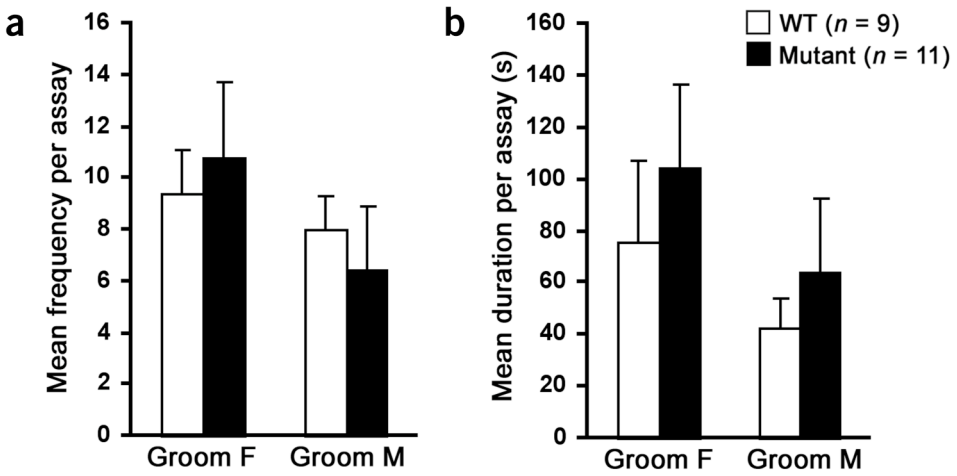
When attacked by a WT intruder the mutant fled or defended himself, suggesting that even under attack mutants are unlikely to initiate fights. Sexually experienced males are more aggressive than naïve males. However, individual housing induces aggression in males independent of mating experience. Our male residents are singly housed for several weeks, making it unlikely that the reduced aggressivity in *Cnga2*$^{-/Y}$ 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.



Supplementary Figure 1: *Cnga2* mutant males groom conspecifics in a manner similar to WT males.

Cnga2 mutant and WT resident males were observed for grooming of intruder females in the mating assay and intruder males in the resident-intruder aggression test. The frequency (**a**) as well as the duration (**b**) of grooming behavior towards the intruder appeared to be similar between WT males and *Cnga2*$^{-/Y}$ (P = 0.97, frequency of grooming female; P = 0.59, duration of grooming female; P = 0.2, frequency of grooming intruder male; P = 0.59, duration of grooming intruder male). Groom F = grooming of WT intruder female by resident male in mating test; Groom M = grooming of WT intruder male by resident male in the resident-intruder aggression test.