

References and Notes

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41. The simulated warming of near-surface air temperatures over Greenland (lower left panel in Fig. 3A) is underestimated (2°C) in the model, as compared to about 7° ± 3°C in the reconstructions. The most likely reason for this model discrepancy is related to an underestimation of the northward heat flux into the Nordic Seas and as a consequence to a cold bias over the Nordic Seas in the interstadial state (a known problem in most coarse-resolution models,

which also occurs in present-day simulations). Hence, warming of the Nordic Seas due to a restart of the THC is underestimated, and the warming in adjacent regions such as Greenland via atmospheric heat transport is also too small.

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Loss of Sex Discrimination and Male-Male Aggression in Mice Deficient for TRP2

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The mouse vomeronasal organ (VNO) is thought to mediate social behaviors and neuroendocrine changes elicited by pheromonal cues. The molecular mechanisms underlying the sensory response to pheromones and the behavioral repertoire induced through the VNO are not fully characterized. Using the tools of mouse genetics and multielectrode recording, we demonstrate that the sensory activation of VNO neurons requires TRP2, a putative ion channel of the transient receptor potential family that is expressed exclusively in these neurons. Moreover, we show that male mice deficient in TRP2 expression fail to display male-male aggression, and they initiate sexual and courtship behaviors toward both males and females. Our study suggests that, in the mouse, sensory activation of the VNO is essential for sex discrimination of conspecifics and thus ensures gender-specific behavior.

Animals have evolved specific communication strategies to identify and attract a mate. Pheromones are a discrete class of chemical cues that signal the sex and the social status of an individual and promote coordinated motor programs and physiological changes essential for breeding and aggression among conspecifics (*J*). The highly reproducible and species-specific character of the response to pheromones offers a valuable experimental system for studying the neural basis of genetically preprogrammed behaviors.

Terrestrial vertebrates have evolved two anatomically distinct sets of olfactory neurons and brain centers to detect and analyze the surrounding chemical world. The main olfactory epithelium (MOE) is located in the posterior recess of the nasal cavity and is accessible to small odorant chemicals carried in the air. The olfactory information detected in the MOE is

transmitted to the main olfactory bulb (MOB). Further sensory processing in distinct centers of the primary olfactory cortex and in multiple cortical and neocortical areas generates in humans the distinctive perception of smell (2). In contrast, neurons of the vomeronasal organ (VNO) are enclosed within a bilateral and tubular-shaped chemosensory structure of the ventral nasal septum (3). In rodents, the VNO opens into the ventral groove of the nasal cavity where it gains access to water-soluble chemical cues carried by the nasal mucus. VNO neurons send fibers to the accessory olfactory bulb (AOB), which in turn projects to discrete loci of the ventromedial hypothalamus via the medio-cortical amygdala (3). Previous work has suggested that the VNO has a primary role in the detection of pheromones and in eliciting behavioral and physiological responses to conspecifics. Surgical ablation of the VNO in rodents, for example, markedly diminishes mating and intraspecies aggressive behavior of the male and impairs pheromone-induced changes in the estrus cycle of the female (3, 4).

How is the pheromone information detected by chemosensory receptors and transduced into electrical activity by sensory neurons? Molecular studies have shown that MOE and VNO sensory neurons use distinct transduction mechanisms to mediate chemosensory signaling (5). In the MOE, specific recognition of odorants is achieved by about 1000 heterotrimeric GTP-

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binding protein (G protein)-coupled odorant receptors (ORs) and is mediated by the activation of a cyclic nucleotide-dependent signal transduction cascade. In contrast, VNO signaling involves several receptor families that are not molecularly related to the ORs (6–11). Moreover, unlike MOE olfactory neurons, VNO neurons do not use cyclic nucleotides as second messengers to transduce pheromone receptor activation (12). Instead, it has been proposed that mammalian pheromone signaling involves a member of the TRP superfamily of ion channels and phospholipase C (PLC) activation (13–15). TRP ion channels are involved in G protein-regulated, cyclic nucleotide-independent transduction pathways in a variety of sensory systems such as mechanosensation and olfaction in the nematode *Caenorhabditis elegans* and phototransduction in the fruitfly *Drosophila* (16, 17). In the *Drosophila* eye, photoisomerization of rhodopsin activates a G α protein of the G α_{cq} class, which in turn triggers a phosphatidylinositol-signaling cascade and leads to the opening of the cation-selective channels dTRP and dTRPL (18). Similarly, a TRP-related protein, TRP2, has been shown to be exclusively expressed by VNO neurons, and inhibition of PLC- β activity eliminates VNO sensory responses (13, 14). The TRP2 protein localizes to VNO sensory microvilli, the proposed site of pheromone sensory transduction, suggesting a direct role of the channel in the pheromone-evoked response. The TRP2 channel might represent the primary conductance activated by pheromone signals, or it could mediate a secondary amplification or modification of the sensory response.

Genetic ablation of TRP2 in the mouse allowed us to investigate the requirement of TRP2 function in VNO signaling and to directly assess VNO-mediated sensory responses and behaviors. We report here that TRP2 deficiency eliminates the sensory activation of VNO neurons by urine pheromones. Moreover, the absence of VNO function has pronounced behavioral effects. TRP2^{-/-} male mice appear unable to recognize the sexual identity of their conspecifics: They fail to display the pheromone-evoked aggression toward male intruders that is normally seen in wild-type males, and they display courtship and mounting behavior indiscriminately toward both males and females. Our data contradict the established notion that VNO activity is required for the initiation of male-female mating behavior in the mouse and suggest instead a critical role in ensuring sex discrimination.

Genetic ablation of TRP2 in the mouse.

The exquisite specificity of TRP2 expression in the VNO provides a valuable experimental tool for genetically modifying VNO function (13, 19). We used gene targeting to construct a mouse line in which expression of the TRP2 protein is abolished. The construct strategy (20) was aimed at deleting functionally critical re-

gions of the protein that include the transmembrane domains 4 and 5, the putative channel pore, and a sequence motif, Glu-Trp-Lys-Phe-Ala-Arg, shared among all TRP genes (13, 21, 22). The linearized targeting construct was electroporated into 129/Sv embryonic stem cells. Clones carrying the targeted allele (20) were injected into C57Bl/6J blastocysts to produce male germ line chimeras that were in turn mated with C57Bl/6J females. The F₁ progeny were mated to generate hetero- and homozygous animals, whose offspring were used for phenotypic analysis. We confirmed by Western blotting that a complete null allele had been generated (20). Casual observation revealed no obvious phenotype in the TRP2 mutants. The TRP2^{+/-} and TRP2^{-/-} mice were similar to wild-type mice in general fitness, average litter size, and longevity. TRP2^{-/-} pups nursed without aid and thrived into adulthood at a rate similar to that of wild-type mice, as assessed by size and age at sexual maturity. TRP2^{-/-} females displayed normal maternal behaviors such as crouching and nursing, and transfer of a nursing female to a new cage initiated nest-building activity and efficient pup retrieval.

Absence of pheromone-evoked neuronal activity in VNOs of TRP2^{-/-} mice. Molecular, imaging, and electrophysiological studies have indicated that the neuronal population of the rodent VNO is highly diverse, expressing as many as 400 putative pheromone receptors (15). The large repertoire of VNO receptors is likely to reflect a similarly large diversity of pheromonal cues. Conventional single- or few-cell recording techniques are therefore likely to be inadequate to sample the range of VNO chemosensory responses. Instead, we analyzed simultaneously the sensory response of large populations of VNO neurons using multielectrode extracellular recording. Two strategies were used to maximize the diversity of the sampled receptor population. We first used a flat array of 61 electrodes, with the neuroepithelium mounted on the array such that the microvillar surface is directly exposed to the chemosensory stimulation (14, 20). In addition, to circumvent a potential bias in the previous technique toward neurons located in the basal VNO neuroepithelium, which express different G proteins and pheromone receptors from those expressed by neurons of the apical region (15), we also recorded with a bundle of 31 wire electrodes penetrating the neuroepithelium from the apical side (20). The analysis was performed on VNOs dissected from male and female TRP2^{+/-} and TRP2^{-/-} mice.

Behaviorally effective pheromones of largely unknown chemical nature are abundant in secretions from the skin and reproductive tract, and in urine. Pheromones present in mouse urine generate a specific increase in the spiking rate of about 40% of VNO neurons recorded with a flat electrode array (14). When tested

repeatedly with pulses of thousandfold-diluted mouse urine, neurons in the VNO of a TRP2^{+/-} mouse (Fig. 1A, left) showed a reproducible increase in firing rate. On average, this electrode recorded a peak 20-Hz increase above baseline in spiking rate for this stimulus. In contrast, the waveform recorded in the VNO of a TRP2^{-/-} mouse showed spontaneous firing but no stimulus-driven change in rate (Fig. 1A, right). To summarize the multiunit response recorded on individual electrodes, we calculated the average change in rate between two time intervals: the 5 s before valve opening and the 5 s after valve opening. The rate increased markedly at the site recorded in the TRP2^{+/-} mouse, but not in the TRP2^{-/-} mouse (Fig. 1A, bottom panels).

We examined the average change in firing rate over time in response to stimuli in individual electrodes as well as averaged across all active recording sites in one preparation (Fig. 1B). In agreement with our previous findings (14), some VNO neurons of TRP2^{+/-} mice responded to diluted urine by increasing their firing rate, typically by up to 30 Hz. Some neurons responded to pheromonal cues from both sexes, whereas others showed selectivity for either male or female urine (Fig. 1B, left). VNO neurons were also stimulated by a brief pulse of potassium, which acts directly on the neuronal membrane, bypassing the chemosensory signal-transduction cascade. In similar recordings from TRP2^{-/-} mice (Fig. 1B, right), VNO neurons failed to respond to any diluted urine stimuli but still responded reliably to the nonspecific high-potassium solution, demonstrating that they were still electrically active and could fire upon depolarization.

Consistent results were obtained across multiple VNO preparations of male and female TRP2^{+/-} and TRP2^{-/-} mice in both flat-array and wire-bundle recordings (Fig. 1C). If we use the criterion that a positive response corresponds to an increase of at least 5 Hz in firing rate, 71 of 160 recording sites in TRP2^{+/-} mice gave responses to at least one of the urine stimuli, whereas none of the 262 recording sites in TRP2^{-/-} mice gave a positive response. Assuming that each of these recording sites represents an independent measurement, we can say with 95% confidence that <1% of the sites in TRP2^{-/-} mice might be responsive and have escaped detection in our experiments. In contrast, 44% of the sites in the TRP2^{+/-} mice are responsive.

These results suggest that, within the limits imposed by the finite size of our recording sample, genetic ablation of the TRP2 channel eliminates the sensory responses of VNO neurons to pheromonal cues present in urine, implying that the TRP2 conductance has an essential role in the transduction of pheromone signals. TRP2 protein could be the primary conductance of the pheromone-evoked response, or it could act as an essential component of a

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multisubunit channel. Members of the TRP family can function both as homomers and as heteromers with the channel properties varying with subunit composition (16, 23–24). Our search for additional members of the TRP family in the VNO has consistently failed (13), suggesting that TRP2 may function alone or in combination with non-TRP-like molecules.

TRP2 is required for the maintenance but not the initial formation of the pheromone receptor map. To evaluate the role of sensory activity in the establishment and maintenance of appropriate VNO axonal connections to the brain, we crossed TRP2^{-/-} mice to mice carrying the *VN12-IRES-tauLacZ* mutation (25). The expression at a particular receptor locus of a bicistronic RNA encoding the pheromone receptor and the fusion protein tau-β-galactosidase (β-Gal) provides a genetic tool to visualize VNO

sensory neuron afferents. The visualization of the axonal projections from specific subsets of VNO neurons showed the presence of topographically conserved domains of axonal projections in the accessory olfactory bulb that differ from those of neurons expressing distinct receptors (25).

Whole-mount X-Gal staining (20) performed on brains dissected from *VN12-IRES-tauLacZ*^{+/-}; TRP2^{+/-} mice at various postnatal and adult stages showed the formation of a complex glomerular map in the anterior AOB (Fig. 2, left). The VN12 receptor maps appear indistinguishable in TRP2^{+/+} and TRP2^{+/-} littermates at all stages analyzed. Similarly, at young postnatal stages, the pattern of projections and the overall number of β-Gal-positive fibers reaching the AOB appear identical in TRP2^{+/-} and TRP2^{-/-} mice (Fig. 2, top). However, a clear difference in the density of

axonal projections emerged in mice aged 1 month and older. The AOB of TRP2^{-/-} mice displayed fewer blue fibers within the vomeronasal nerve and the glomeruli in comparison with TRP2^{+/-} littermates, although the projection pattern remained identical (Fig. 2). Owing to allelic exclusion of pheromone receptor genes, mice homozygous for the *VN12-IRES-tauLacZ* mutation display twice as many X-Gal-positive neurons and fibers as do mice heterozygous for this mutation. The difference in the density of X-Gal staining in the AOBs of TRP2^{+/-} and TRP2^{-/-} mice appeared very similar to the difference between *VN12-IRES-tauLacZ* homozygous and heterozygous mice. Thus, loss of TRP2 function appears to reduce the number of axonal projections by about twofold.

These results suggest that TRP2-mediated sensory activity of VNO neurons is required for

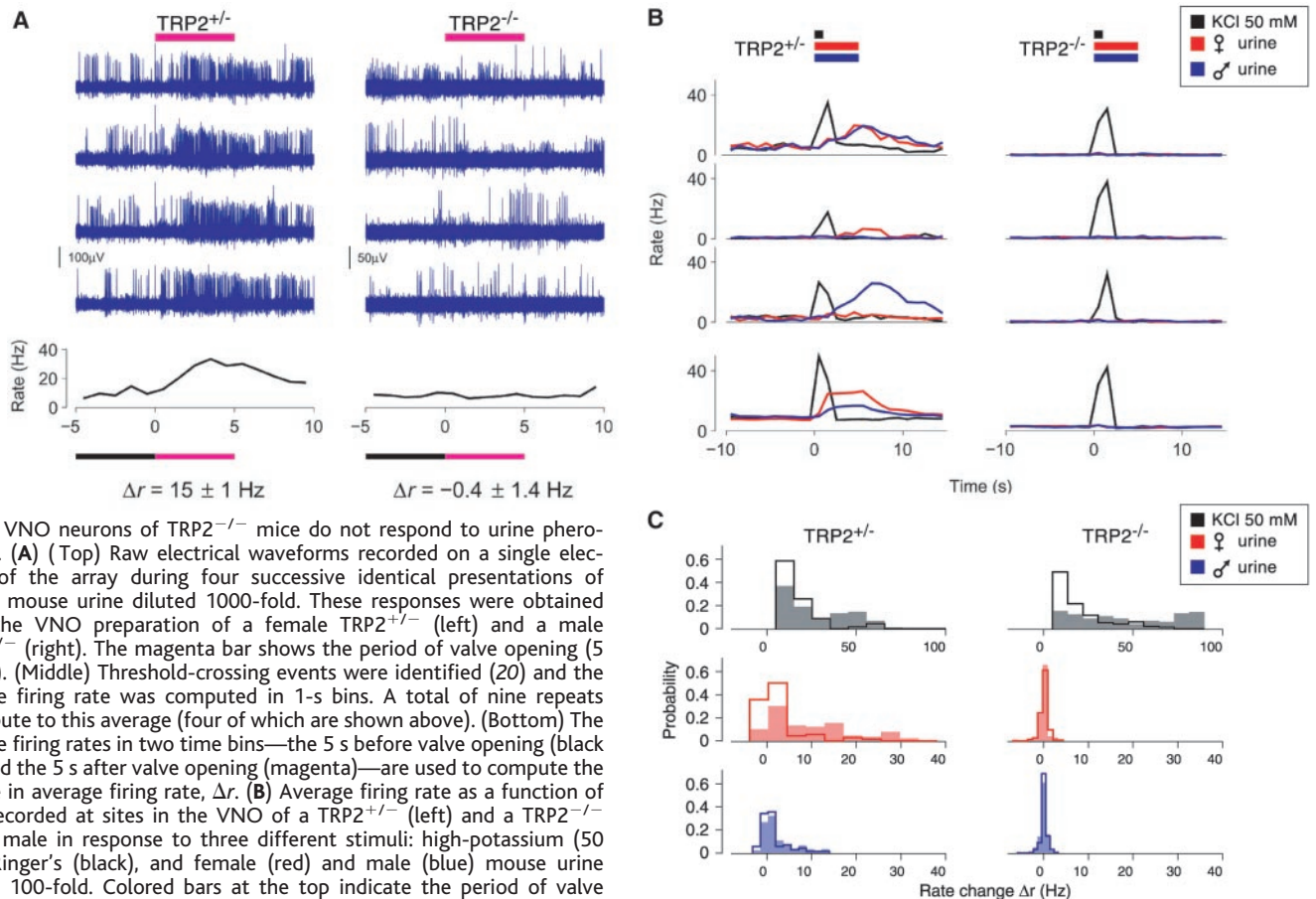


Fig. 1. VNO neurons of TRP2^{-/-} mice do not respond to urine pheromones. (A) (Top) Raw electrical waveforms recorded on a single electrode of the array during four successive identical presentations of female mouse urine diluted 1000-fold. These responses were obtained from the VNO preparation of a female TRP2^{+/+} (left) and a male TRP2^{-/-} (right). The magenta bar shows the period of valve opening (5 s total). (Middle) Threshold-crossing events were identified (20) and the average firing rate was computed in 1-s bins. A total of nine repeats contribute to this average (four of which are shown above). (Bottom) The average firing rates in two time bins—the 5 s before valve opening (black bar) and the 5 s after valve opening (magenta)—are used to compute the change in average firing rate, Δr . (B) Average firing rate as a function of time recorded at sites in the VNO of a TRP2^{+/+} (left) and a TRP2^{-/-} (right) male in response to three different stimuli: high-potassium (50 mM) Ringer's (black), and female (red) and male (blue) mouse urine diluted 100-fold. Colored bars at the top indicate the period of valve opening: 1 s for the potassium solution and 5 s for the pheromonal stimuli. Each stimulus was repeated nine times. The top three pairs of panels show examples of firing rates recorded at three sites in a single preparation, whereas the bottom pair shows the average rate over all potassium-responsive recording sites [for each preparation, 35 sites in TRP2^{+/+} and 45 in TRP2^{-/-} (20)]. (C) The distribution of Δr , the average firing-rate change upon stimulation. The stimuli were identical to those used in (B). Histograms of Δr from array recordings are shown in solid bars with light colors, whereas results from wire-bundle recordings are in outline bars with bold color. These histograms show combined data from all active sites (20) in several preparations. In array recordings of three preparations of TRP2^{+/+} mice containing 90 active sites, 53 sites displayed an increase in firing rate of ≥ 5 Hz in response to female mouse pheromones, and 20 sites

showed responses to male mouse pheromones. By contrast, recordings from TRP2^{-/-} mice showed responses to the high-potassium solution similar to those of TRP2^{+/+}, but showed no change in firing rate in response to pheromonal stimuli; in five experiments comprising 105 active recording sites, we consistently failed to detect any increase in firing rate >5 Hz in response to male or female urine pheromones. Similar results were obtained when we recorded with a bundle of 31 wire electrodes inserted from the apical side of the tissue. The open bar summarizes the results from a total of 70 active sites in three TRP2^{+/+} preparations and 157 active sites in four TRP2^{-/-} preparations. In TRP2^{+/+} mice, 17 sites showed responses >5 Hz to either female or male urine. In the TRP2^{-/-} mice, none of the recording sites showed responses to urine stimuli.

the maintenance but not the initial formation of the pheromone receptor map. Further evidence of this was obtained by *in situ* hybridization in the VNO itself. Using RNA probes for pheromone receptors and for the two $G\alpha$ protein subunits expressed by VNO neurons, we detected a partial loss of mature sensory neurons in the adult VNO (20). We estimate that, at 2 months of age, TRP2^{-/-} mice have 50% fewer V1R-V3R neurons and 75% fewer V2R neurons than TRP2^{-/-} mice of comparable age.

The premature death of some VNO neurons in the absence of sensory activity contrasts with the apparently normal survival of MOE neurons in mice mutant for the gene encoding the α subunit of the olfactory cyclic nucleotide-gated channel (OCNC1) (26, 27). In the MOE, the requirement of sensory activity for neuronal survival has only been observed in mosaic epithelia in which both sensory silent and active neuronal populations are present. This situation generates a selective disadvantage for neurons lacking OCNC1 expression, presumably because of a reduced access to trophic factors delivered by activated target neurons (28).

The mating behavior of TRP2^{-/-} male mice toward females appears normal. The VNO has been implicated in the initiation of sexual behaviors in male rodents. Most notably, the surgical ablation of the VNO in sexually naïve hamsters was found to abolish male sexual behavior (29, 30). Similar experiments with mice suggest a significant although much less marked reduction in male mounting frequency (31, 32). Inactivation of the TRP2 gene provides a valuable opportunity to analyze the behavior of animals genetically deprived of VNO function.

The mating behavior of sexually naïve TRP2^{-/-} males toward females was investi-

gated in several ways. We first monitored the production of vaginal plugs in either wild-type C57Bl/6J or TRP2^{-/-} females after they had been housed overnight with a TRP2^{-/-} male. The production of plugs by TRP2^{-/-} males occurred at a frequency indistinguishable from that of wild-type or TRP2^{+/-} males, indicating that TRP2 function is not essential for male-female mating. This conclusion was reinforced by a more detailed analysis of the sexual behavior of TRP2^{-/-} ($n = 6$), TRP2^{+/-} ($n = 6$), and wild-type ($n = 4$) males with video recordings (20). The three groups of mice showed no difference in either the mean number of mounts over a 15-min observation interval (TRP2^{-/-} = 10.2 ± 2.6 ; TRP2^{+/-} = 12.1 ± 4.1 ; TRP2^{+/+} = 9.0 ± 2.3 ; mean \pm SEM) or in the overall duration of mounting episodes (TRP2^{-/-} = 44 ± 12 s; TRP2^{+/-} = 66 ± 22 s; TRP2^{+/+} = 30 ± 13 s). This analysis strongly suggests that sexually naïve TRP2^{-/-} males display innate sexual behavior that is indistinguishable from that observed in males with a fully functional VNO.

Pheromone-evoked male-male aggression is abolished in TRP2^{-/-} mice. We next evaluated the importance of VNO function in male-male intraspecies aggression, which is evoked by chemical cues in male urine and which is thought to require VNO activity (33). We monitored the ability of TRP2^{-/-} males to initiate aggressive displays in response to pheromones using the resident-intruder paradigm (34, 35). No conditioning or training period is necessary in this assay as it relies exclusively on innate and stereotyped behaviors initiated by pheromones. After an isolated resident male mouse has established the cage as its territory, another male is in-

troduced into the cage as an intruder. Under normal circumstances this elicits aggressive behavior from the resident that is triggered by pheromones in the intruder's urine (34) and that relies on testosterone production by both the resident and the intruder. Indeed, castrated males do not display aggressive behavior and do not evoke aggression by the resident, whereas the swabbing of sexually mature male urine on the back and urogenital area of a castrated male initiates robust aggression from the resident (36). The use of a castrated male intruder ensures that any aggression observed in the assay originates from the resident, not the intruder (35), and is unambiguously mediated by swabbed pheromone cues.

Upon reaching sexual maturity, male mice were individually caged for 7 to 10 days before the behavioral tests (20). Aggressive behavior by the resident was monitored in two separate 10-min trials, first after a castrated male intruder was introduced into the resident cage, and then after freshly collected male urine had been swabbed on the back and urogenital region of the same castrated intruder (Fig. 3A). In the first trial, wild-type and TRP2^{+/-} residents were only minimally aggressive (average total attack duration over the 10 min period: wild type = 6 ± 5 s, $n = 17$; TRP2^{+/-} = 9 ± 4 s, $n = 25$), whereas TRP2^{-/-} mice showed no signs of aggression (average attack duration = 0, $n = 27$). When the intruder was swabbed with male urine, both the wild-type (average attack duration = 83 ± 19 s, $n = 17$) and TRP2^{+/-} (average attack duration = 44 ± 9 s, $n = 25$) resident mice displayed robust aggressive behavior, starting with threatening displays such as tail rattling and piloerection, and evolving into offensive attacks upon the intruder. Remarkably, the swabbed intruder did not evoke aggression from any of the resident TRP2^{-/-} males (attack duration = 0, $n = 27$), nor was there any evidence of overt agitation such as displacement digging or self-grooming (20).

The absence of pheromone-evoked aggression in the TRP2^{-/-} males could be a direct consequence of the inability of VNO neurons to provide the brain with sensory information or it could result from a more indirect effect of the TRP2 mutation on brain centers involved in aggressive behaviors. We therefore performed several experiments to ensure that the mutant mice were physically able to display aggressive behaviors.

We first investigated the production of testosterone by TRP2^{-/-} males (20). It has been shown that aggressive behavior is impaired in male mice deficient in testosterone production or detection and that normal aggressive behavior can be restored in castrated males by injection of testosterone (37, 38). To test whether testosterone production was altered in the TRP2^{-/-} males, we measured serum levels of testosterone by radioimmunoassay (RIA) (Fig.

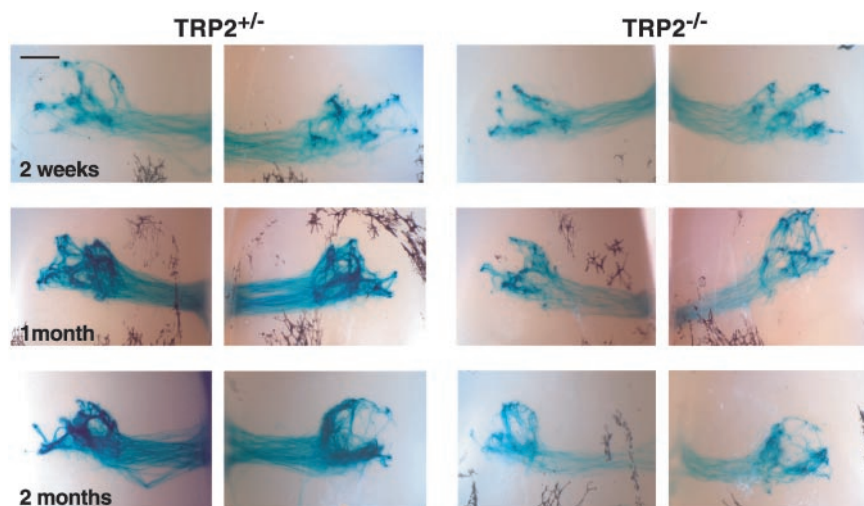


Fig. 2. VNO neuronal projections and receptor survival in TRP2^{+/-} and TRP2^{-/-} mice. Visualization of the sensory projection of neurons expressing the VN12 pheromone receptor in the cross of the TRP2 mutants with the *VN12-ires-tauLacZ* mutant line. The panel pairs show the left and right bulbs of the same mouse. At 2 weeks postnatally, the number of VN12 projections to the AOB appears identical in the TRP2^{+/-} and TRP2^{-/-} mice. However, the pattern of β -Gal-positive fibers in mice aged 1 month and older suggests a loss of sensory afferents in the TRP2^{-/-} mutant (27). Bar, 100 μ m.

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3C). This analysis revealed that whereas testosterone production was abolished in control wild-type castrated males (0 ng/ml, $n = 2$), in the TRP2^{-/-} mice (5.15 ± 1.34 ng/ml, $n = 11$) testosterone levels were equal to or even slightly greater than those of wild-type (2.77 ± 1.12 , $n = 8$) and TRP2^{+/-} mice (2.60 ± 1.33 ng/ml, $n = 8$).

To further control for the ability of TRP2^{-/-} males to display nonpheromone-driven aggressive behaviors, we introduced sexually mature TRP2^{-/-} males into the cages of wild-type resident males. As expected, the presence of a TRP2^{-/-} male intruder triggered attacks from the wild-type resident. Faced with the aggressive behavior and the attacks of the resident males, the TRP2^{-/-} intruders responded by initiating threatening aggressive displays and counterattacks ($n = 4$) (39). These results indicate that the TRP2^{-/-} males are physically and neurologically capable of displaying aggressive behavior but are not triggered to do so by pheromonal cues.

Finally we tested whether the TRP2 mutation might exert its effects through a change in the main olfactory system. We first evaluated whether the MOE of TRP2^{-/-} males remains functional by hiding a small piece of cookie under pine shavings in a large clean cage (40, 41) and monitoring the time required for the mice to find the cookie. The mice had not been previously trained in this assay; however, food was removed 48 hours before testing to increase motivation. The time required to find the cookie was similar for TRP2^{+/+} (100.5 ± 18 s, $n = 6$), TRP2^{+/-} (92.5 ± 23 s, $n = 6$), and TRP2^{-/-} mice (86.9 ± 15 s, $n = 9$), suggesting that the mutation does not disrupt MOE function (Fig. 3D).

Information about MOE function was also obtained from the resident/intruder aggression assay. Wild-type male residents spend a large part of the assay sniffing the head, back, and urogenital area of the intruder. This sniffing behavior is abolished by ablation of the olfactory bulbs (33). We therefore scored in each assay the amount of time that the resident male spent sniffing the intruder (Fig. 3D). The wild-type (79 ± 19 s, $n = 17$) and TRP2^{+/-} mice (83 ± 15 s, $n = 25$) spent ~20% of the assay sniffing the intruder swabbed with male pheromone (Fig. 3D), whereas the TRP2^{-/-} resident mice spent over 30% of the assay sniffing the intruder (126 ± 14 s, $n = 27$) (Fig. 3D). This difference may be attributable to the inability of wild-type males to engage in “neutral” sniffing behavior while initiating aggressive display. Together, these behavioral assays suggest that the main olfactory system was not impaired by the TRP2 mutation and that the observed phenotypes are due specifically to disruption of the vomeronasal sensory system.

TRP2^{-/-} male mice display sexual behavior toward male intruders. The TRP2^{-/-} males displayed a pronounced and unexpected

phenotype in the resident/intruder assay. Instead of attacking the intruder male, the sexually naïve TRP2^{-/-} resident male approached the intruder and engaged in sexual behavior (mounting and thrusting) that resembled the sexual behavior normally directed toward female mice (Fig. 4, A and B) (20). Although

under certain experimental conditions, some strains of male mice display occasional mounting activity with animals of the same gender (42), the mounting behavior observed in our study toward the intruder was displayed exclusively by TRP2^{-/-} males. This mounting behavior (Fig. 4B) was directed toward castrated

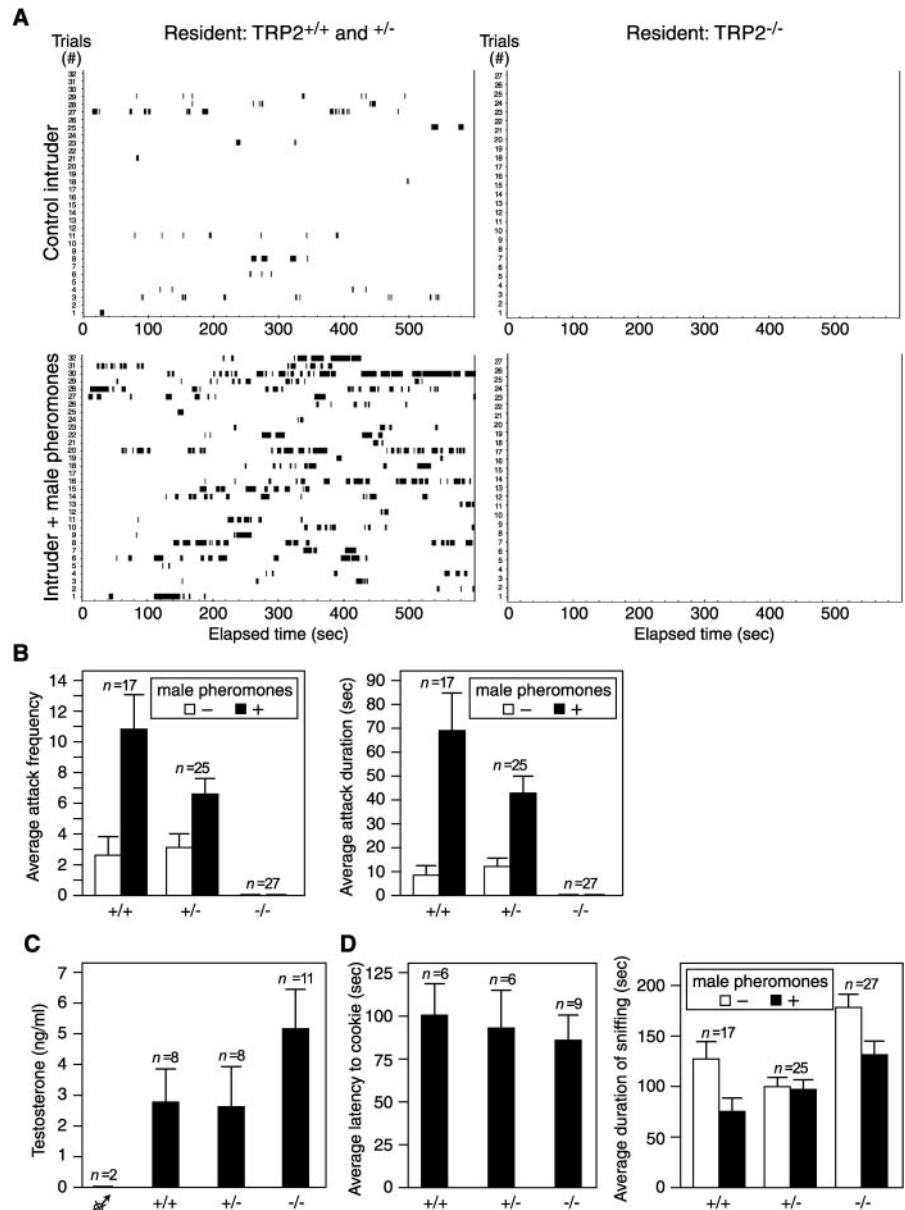


Fig. 3. TRP2^{-/-} mice do not display male-male aggression in a resident/intruder assay. (A) Attack behavior of TRP2^{+/+}, TRP2^{+/-} and TRP2^{-/-} mice (20). Raster plots depict resident attacks during individual trials [supplemental video analysis (20)]. Each black tick indicates one attack, and the width of the tick indicates the duration of the attack. In the upper panels the intruder is a castrated male; in the lower panels the castrated male intruder is swabbed with urine from sexually mature mice. Left panels are TRP2^{+/+} (trials 1 to 17) and TRP2^{+/-} mice (trials 18 to 32); right panels are TRP2^{-/-} mice (trials 1 to 27). (B) Quantification of resident average attack frequency and duration extracted from the data shown in (A). Open bars represent the intruders not swabbed with male urine; shaded bars represent the intruders swabbed with male urine. (C) Serum testosterone RIA (20). Castrated mice [male symbol (♂) with X] do not produce testosterone. The amount of serum testosterone produced by TRP2^{-/-} mice is similar to or greater than that produced by TRP2^{+/+} mice. (D) The function of the main olfactory system in TRP2^{-/-} mice appears normal. The time required to discover a hidden cookie (latency) is similar in mutant and wild-type mice (left), as is the sniffing behavior of the two groups of mice during the resident/intruder assays (right).

male intruders whether or not they had been swabbed with male urine pheromones (no pheromone, 15.0 ± 5 s; swabbed pheromone, 28.5 ± 6 s), suggesting that it was triggered by testosterone-independent sensory cues.

Male mice emit ultrasonic vocalizations in response to female cues. These vocalizations are suppressed in the presence of other adult males (43). To evaluate whether the response of the TRP2^{-/-} males toward the intruder was limited to a reflex mounting display, we investigated another aspect of the male mating behavior: We introduced a female or a urine-swabbed castrated male intruder into the resident cage assays while recording vocalizations with a microphone sensitive to ultrasonic frequencies (20). In the presence of a female, the wild-type, TRP2^{+/-} and TRP2^{-/-} resident males emitted robust ultrasonic vocalizations in the 55- to 110-kHz frequency band (Fig. 5, A and B). Wild-type and TRP2^{+/-} resident males emitted no vocalizations in response to male pheromonal cues, consistent with previous reports (42). Remarkably, the TRP2^{-/-} resident males also produced high-frequency vocalizations in response to castrated males swabbed with pheromonal cues from sexually mature males (Fig. 5, A and B). These data suggest that, when exposed to sensory cues from sexually mature males, TRP2^{-/-} males initiate sexual as well as other components of the mouse courtship behavior that, in wild-type mice, have been shown to be triggered exclusively by cues from females.

TRP2^{-/-} mouse mating behavior lacks gender discrimination. There are several possible interpretations for the sexual and courtship behaviors displayed by the TRP2^{-/-} males. The absence of VNO function may generate animals with an irrepressible sexual drive, resulting in mating displays by the mutant toward males if female partners are unavailable. Alternatively, TRP2^{-/-} males may have altered sexual preference such that TRP2^{-/-} males mate with females only in the absence of males. Finally, the TRP2^{-/-} males may be unable to distinguish males from females and thus engage indiscriminately in sexual behavior with a conspecific of either gender. We directly investigated these possibilities by simultaneously introducing into the cage of the TRP2^{-/-} male resident an estrus female and a castrated animal swabbed with male urine. In this assay, the TRP2^{-/-} males consistently mounted both males and females with equal frequency during individual trials (total mounts with castrated male swabbed with male urine: 37 ± 4 ; total mounts with female: 44 ± 5 ; $n = 8$) (Fig. 5C). This assay provides strong evidence that, in the absence of a functional VNO, TRP2^{-/-} males cannot discriminate males from females and will initiate sexual behavior with either gender.

The behavioral phenotype observed in TRP2^{-/-} males clearly differs from that described in published reports of surgical ablation

of the VNO. In those studies, mice showed a decrease in mounting frequency, resulting in significant but not complete disruption of male/female mating behavior (4). In contrast to surgical ablation, genetic targeting readily and specifically eliminates VNO function from birth. Moreover, surgery is invasive and of variable efficiency and specificity. This is of particular concern when surgery is performed on the VNO neuroepithelium, which has self-renewal capacity and is enclosed within a bony capsule (1). Confirmation of other behavioral and endocrine responses ascribed to the activity of the VNO in the mouse, particularly in females, will require further analysis of the TRP2 mutant.

Differences observed with other species of rodents are more difficult to interpret. When the VNO is removed from sexually inexperienced hamsters, male-female mating behavior is entirely abolished in subsets of animals (29, 30). This would suggest that the physiological role of the VNO in controlling animal behavior is variable, even among closely related rodent species. Furthermore, our results are unlikely to

apply to more distant species of mammals or terrestrial vertebrates in which very distinctive and species-specific communication strategies for mate selection have been identified. In birds, for example, auditory cues play an essential role in the identification of an appropriate mating partner, whereas chemosensory signals are unlikely to be behaviorally relevant (44). Importantly, anatomical and molecular evidence suggests that VNO function is absent in higher primates and humans (45). Various investigations have indeed failed to identify sensory neurons in the adult human VNO. In addition, most human pheromone receptor genes appear nonfunctional (46), and sequences from multiple independent samples have demonstrated that the human TRP2 gene is a pseudogene displaying a large number of deleterious mutations in its coding sequence (15). In higher primates and in humans, the evolution of alternative sensory modalities and, in particular, the increased reliance on visual cues (47) parallels a relative decrease in the importance of chemosensory communication.

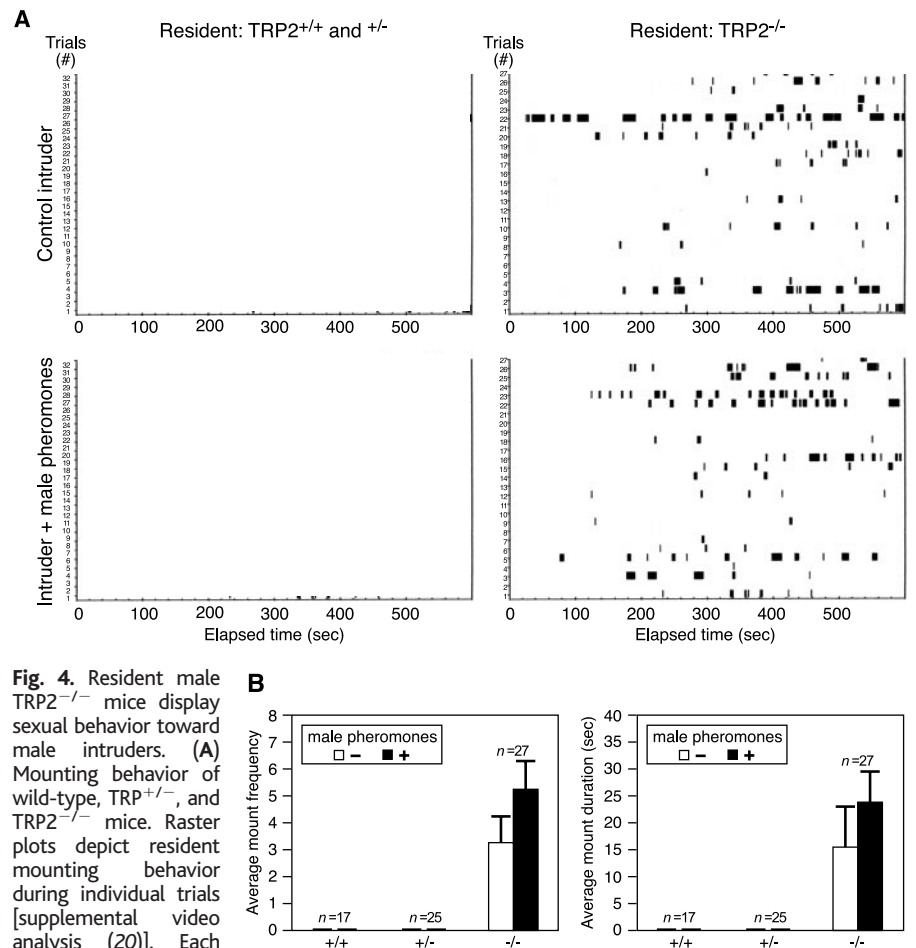


Fig. 4. Resident male TRP2^{-/-} mice display sexual behavior toward male intruders. (A) Mounting behavior of wild-type, TRP2^{+/-}, and TRP2^{-/-} mice. Raster plots depict resident mounting behavior during individual trials [supplemental video analysis (20)]. Each black tick indicates an instance of mounting behavior, and the width of the tick indicates the duration of the behavior. Trials are the same as those shown in Fig. 3A; however, each trial is now scored for mounting activity. (B) Quantification of resident average mounting frequency and duration extracted from data in (A). Open bars represent the intruders not swabbed with male urine; shaded bars represent the intruders swabbed with male urine.

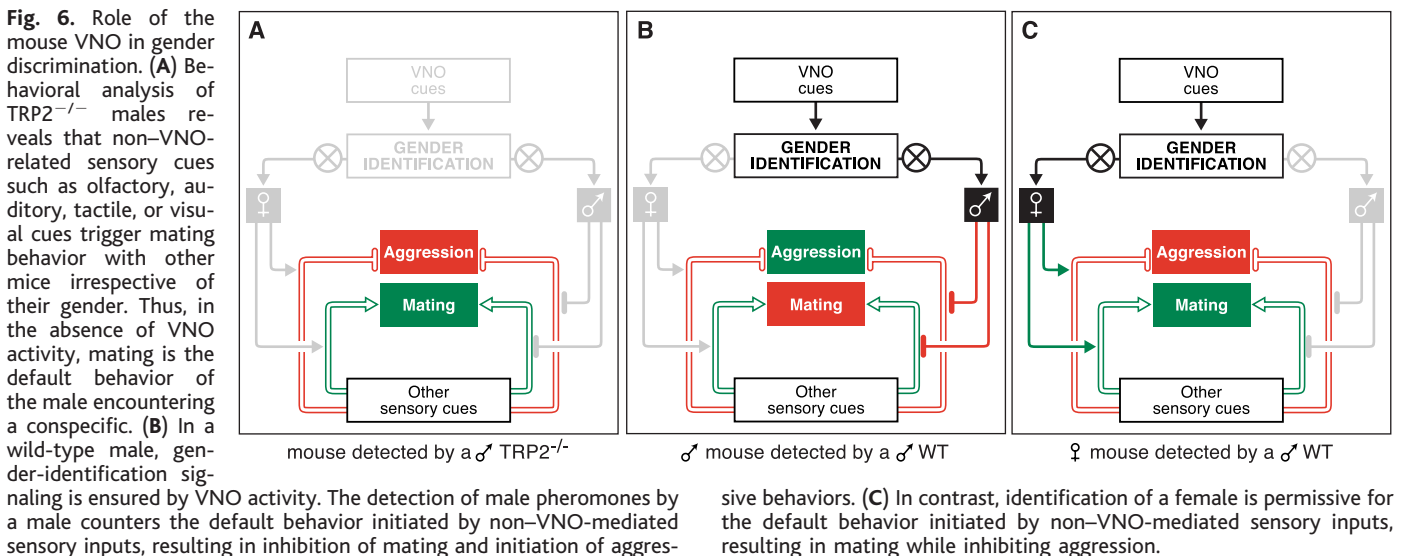
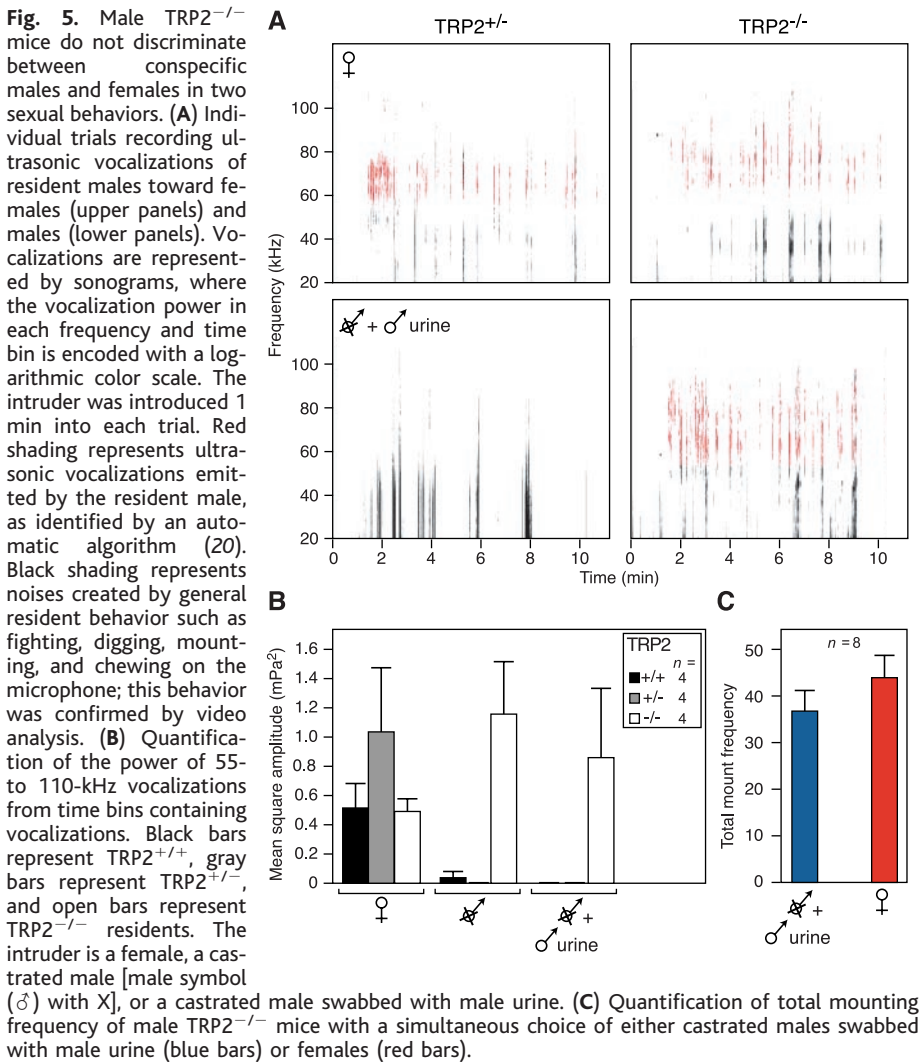
Discussion. We have demonstrated that, in the absence of TRP2 expression, VNO neurons are electrically active but are unable to respond to urine pheromones. Moreover, within the limit of our anatomical analysis,

the development of VNO sensory projections to the AOB of TRP2^{-/-} mice appears normal, suggesting that the behavioral phenotype of the TRP2^{-/-} mutant is likely to result from the lack of VNO sensory activity rather

than from a gross abnormal wiring of the VNO to the brain.

Our results establish the critical role of the mouse vomeronasal system in sex discrimination leading to gender-specific behavior (Fig. 6). The ability of the TRP2^{-/-} male to mate normally with a female indicates that VNO function is not required for initiating sexual behavior. Instead, we show that in TRP2^{-/-} males, non-VNO sensory organs stimulated by the presence of another mouse, irrespective of its sex, elicit mating. Thus, it appears that in the absence of information provided by the VNO, the detection of a conspecific elicits mating as the default behavior (Fig. 6A). Our experiments do not address the nature of the non-VNO sensory cues that elicit mating, but it will be interesting to see whether these cues include olfaction, and whether they are sufficient to restrict the species specificity of the sexual behavior. Moreover, we can conclude that, at least in the sexually naïve mouse, these non-VNO sensory cues are gender-neutral, i.e., either mice fail to identify dimorphic differences in smell, sight, and sounds between a male and a female, or they fail to use this information to control behavioral outputs such as mating that are the most dependent on sex discrimination. Instead, this role appears to be reserved for the vomeronasal system (Fig. 6, B and C). Our data suggest that in the male, detection of male cues by the VNO inhibits the default mating behavior initiated by other sensory cues and instead triggers aggression (Fig. 6B). The behavior of the TRP2^{-/-} mice suggests that appropriate male-male aggressive display is also regulated by VNO function. In contrast, detection of female cues by the VNO appears permissive to the mating behavior triggered by other sensory cues (Fig. 6C).

We propose that a major function of the VNO is to ensure the gender specificity of male mouse behavior by providing the brain with sensory cues that are essential for sex discrimination. Although most of the pheromones



emitted by the mouse remain chemically unidentified, our data suggest that they are not simple "releasers" of motor programs but that they act, at least in part, as regulators of inputs from other sensory organs. This raises two essential questions whose answers will require further insight into the function and the development of the vomeronasal pathway: (i) How is the information about the sexual identity of a conspecific processed by the brain, and how does it control animal behavior? (ii) How and when is the recognition of sex acquired by the brain, and what are the respective roles of genetic and extrinsic determinants in this developmental process?

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19. Our previous studies determined that, in the rat, rTRP2 is exclusively expressed in the microvilli of VNO sensory neurons (13). Recent investigation (48) has confirmed the expression of TRP2 in the mouse VNO, although some studies reported the presence of TRP2 transcript in testes (21, 22). However, our own studies, including immunohistochemistry on tissue sections with a polyclonal antibody directed against the COOH-terminus of Trp2 (13), confirmed the exclusive expression of TRP2 in the VNO but failed to detect any signal in testes (39).
20. The following supplementary material, presented as a description, figure, or movie, is available on Science Online at www.sciencemag.org/cgi/content/full/1069259/DC1: supplementary fig. 1, construction of TRP2^{-/-} mice; supplementary fig. 2, VNO receptor survival in TRP2^{+/-} and TRP2^{-/-} mice; supplementary materials and methods for construction of TRP2^{-/-} mice, Western blot analysis, electrophysiology, electrophysiology data analysis, in situ and histological analysis, mating behavior, male-male aggression, testosterone RIA analysis, and ultrasonic vocalization analysis.
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REPORTS

A Rational Chemical Synthesis of C₆₀

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Isolable quantities of C₆₀, the smallest stable fullerene, have been synthesized in 12 steps from commercially available starting materials by rational chemical methods. A molecular polycyclic aromatic precursor bearing chlorine substituents at key positions forms C₆₀ when subjected to flash vacuum pyrolysis at 1100°C. No other fullerenes are formed as by-products. The methods we have developed for the target-specific synthesis of fullerenes, applied here to a synthesis of C₆₀, should make possible the directed laboratory preparation of other fullerenes as well, including those not accessible by graphite vaporization.

Although C₆₀ and some of the higher fullerenes can be readily prepared by resistive heating of graphite (1), there is still considerable interest in the rational stepwise synthesis of these geodesic all-carbon compounds in isolable quantities. Such approaches may allow derivatives of fullerenes to be prepared and may also provide access to fullerenes that

are not obtained through the uncontrolled vaporization of graphite.

We recently showed that hydrocarbon **1**, which incorporates all 60 carbon atoms, 13 of the 20 benzene rings, and 75 of the 90 carbon-carbon bonds required to form C₆₀, can be "stitched up" with a high-energy laser to generate C₆₀ (Fig. 1) (2). That method, how-

ever, did not permit isolation of the fullerene produced. We have therefore drawn on our experience with the synthesis of smaller geodesic polyarenes, which taught us that incorporation of halogen atoms at strategic sites on polycyclic aromatic hydrocarbons invariably improves the preparative yields of such high-energy reactions, often by two orders of magnitude or more (3–5). We now report that preparative-scale flash vacuum pyrolysis (FVP) of the chlorinated hydrocarbon **2** at 1100°C (6, 7) does, in fact, induce the desired carbon-carbon bond-forming reactions, stitching together the arms of the molecular precursor according to plan and forming the all-carbon polyhedron (Fig. 1). No C₇₀ or any other fullerenes would be expected from such a process, and none are detected in the syn-

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