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A functional circuit underlying male sexual behaviour in the female mouse brain

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In mice, pheromone detection is mediated by the vomeronasal organ and the main olfactory epithelium. Male mice that are deficient for Trpc2, an ion channel specifically expressed in VNO neurons and essential for VNO sensory transduction, are impaired in sex discrimination and male–male aggression. We report here that *Trpc2*$^{-/-}$ female mice show a reduction in female-specific behaviour, including maternal aggression and lactating behaviour. Strikingly, mutant females display unique characteristics of male sexual and courtship behaviours such as mounting, pelvic thrust, solicitation, anogenital olfactory investigation, and emission of complex ultrasonic vocalizations towards male and female conspecific mice. The same behavioural phenotype is observed after VNO surgical removal in adult animals, and is not accompanied by disruption of the oestrous cycle and sex hormone levels. These findings suggest that VNO-mediated pheromone inputs act in wild-type females to repress male behaviour and activate female behaviours. Moreover, they imply that functional neuronal circuits underlying male-specific behaviours exist in the normal female mouse brain.

Males and females within a given animal species display identifiable differences in behaviours, mostly but not exclusively pertaining to sexual and social responses. Although these represent the most obvious examples of behavioural variability within a species, the basic principles underlying sexual dimorphism of brain function are largely unknown. Moreover, with few exceptions, the search for unique structures and circuits in male and female brains that parallel the dimorphism of peripheral sexual organs has so far met little success[1–5].

In many animals species- and sex-specific behaviours are orchestrated by pheromonal cues. Recent studies in rodents have uncovered the dual role of the main olfactory epithelium and the vomeronasal organ (VNO) in pheromones controlling mating, aggression and gender identification[6]. Genetic ablation of the TRPC2 channel, a signalling component essential to VNO function, leads to indiscriminate courtship and mounting behaviour of *Trpc2*$^{-/-}$ male mice towards both males and females, suggesting an essential role of the vomeronasal system in sex identification[7–10]. Furthermore, recent recording, genetic silencing and tracing experiments in the mouse have revealed the involvement of the main olfactory epithelium and associated central pathways in pheromone-mediated responses[11–14].

To study the role of the VNO in female sexual receptivity, we introduced a sexually experienced male to the home cage of either *Trpc2*$^{+/+}$, *Trpc2*$^{+/-}$ or *Trpc2*$^{-/-}$ females. As expected, oestrous *Trpc2*$^{+/+}$ and *Trpc2*$^{+/-}$ females were sexually receptive, allowing intensive olfactory investigation of the anogenital region by the male, leading to successful mating within minutes. However, in a striking role reversal, *Trpc2*$^{-/-}$ females were observed intensively investigating the anogenital region of the intruder males and vigorously attempting to mount them, eliciting aggressive responses from the males.

Male-like behaviours of *Trpc2*$^{-/-}$ females

Female–female and female–male mounting in rodents has been observed mainly in laboratory rats as part of dominance or sexual solicitation, respectively[15–17]. The behaviour observed in *Trpc2*$^{-/-}$ females may thus represent either the exaggeration of normal female responses, or abnormal male-like displays. We monitored unique characteristics of male sexual and courtship behaviours in *Trpc2*$^{+/+}$, *Trpc2*$^{+/-}$ and *Trpc2*$^{-/-}$ male and female residents towards female and male intruders. To avoid the aggressive behaviour of wild-type males while controlling for the presence of pheromones, castrated or bulbectomized male swabbed with male urine were used as male intruders.

Male-like sexual display was investigated by scoring the number of animals mounting the intruder (Fig. 1a), the average duration of mounting (Fig. 1b) and the latency (time taken) to mount (Supplementary Fig. 1a) in a 15 min assay. Because mounting can include aspects of dominance, we also monitored occurrences of pelvic thrusts as a more stringent criteria of sexual behaviour (Fig. 1c). Results from all four tests demonstrate that *Trpc2*$^{+/+}$ and *Trpc2*$^{+/-}$ females very rarely displayed characteristics of male-like sexual behaviour towards female intruders, while the majority of *Trpc2*$^{-/-}$ females (Supplementary Video 1), *Trpc2*$^{+/-}$ and *Trpc2*$^{-/-}$ males showed robust mounting, pelvic thrusts and a short latency to mount. Remarkably, the behaviour of *Trpc2*$^{-/-}$ females towards other females was statistically indistinguishable from that of heterozygous and mutant males. Analysis of the response to male intruders showed that only *Trpc2*$^{-/-}$ males and females (Supplementary Video 2) displayed significant levels of male-like sexual behaviour towards males, and that their behaviour is both statistically indistinguishable from each other, and from their response to female intruders

We further assessed male-specific courtship behaviours. Male mice engage and solicit females by raising the female rear with their snout. Also, when interacting with females, adult males emit ultrasonic vocalizations at high (30–110 kHz) frequencies, while adult females produce only a limited range of ultrasounds during female–female social investigation[18–22]. In addition, males perform intense olfactory investigation of the female rear, while females focus on the head and body.

The scoring of solicitation (Fig. 1d), ultrasound duration and complexity, latency to whistle, number of animals emitting ultrasounds (Fig. 1e, Fig. 2, Supplementary Fig. 1b–d), and olfactory

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investigation of anogenital region (Supplementary Fig. 1e) further confirmed that the behaviour of *Trpc2*$^{-/-}$ females towards females cannot be distinguished from that of *Trpc2*$^{-/-}$ and *Trpc2*$^{+/-}$ males, and is very different from that of *Trpc2*$^{+/+}$ and *Trpc2*$^{+/-}$ females. Moreover, the behaviour of *Trpc2*$^{-/-}$ males and females towards male and female intruders was similar and, when intruders were presented simultaneously, *Trpc2*$^{-/-}$ females, as previously shown with *Trpc2*$^{-/-}$ males[9,10], display no preference for either sex, engaging indiscriminately in sexual behaviour with both male and female intruders with equal frequency ($n = 6$, data not shown).

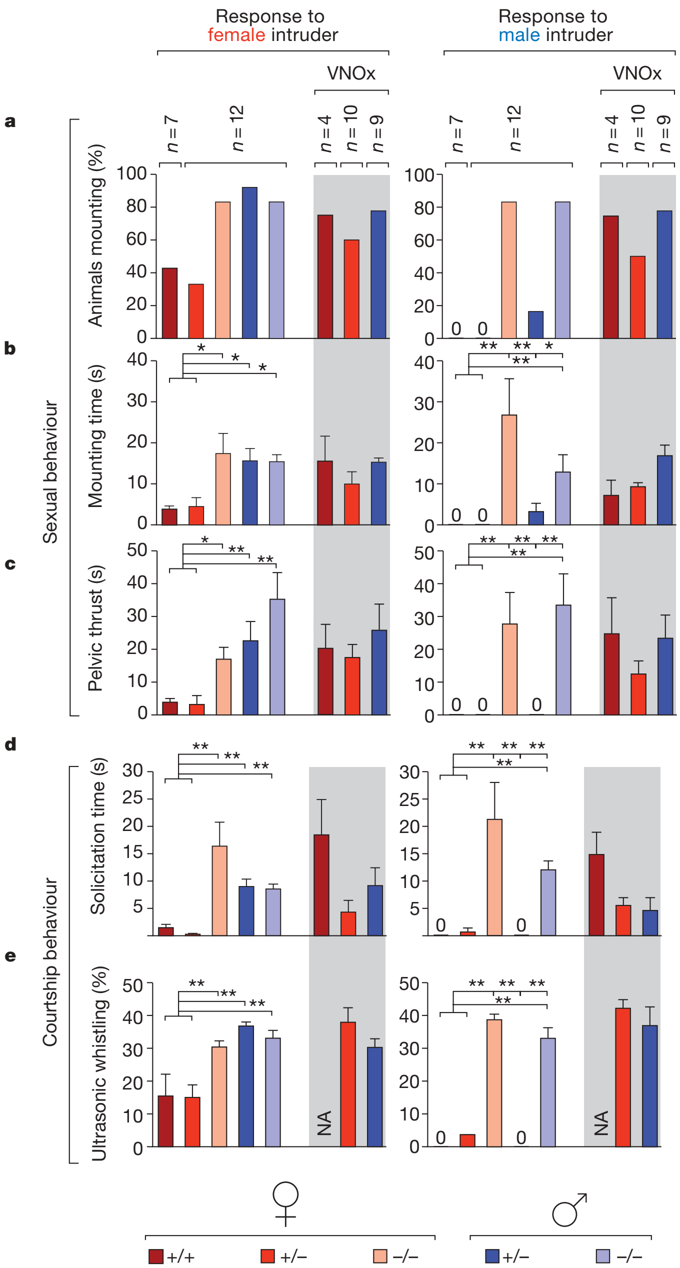

Figure 1 | Male-like sexual and courtship behaviours are displayed by *Trpc2*$^{-/-}$ and VNOx females. Sexual (**a**–**c**) and courtship (**d** and **e**) behaviours towards female (left) and male (right) intruders observed in sexually naive adult *Trpc2*$^{+/+}$, *Trpc2*$^{+/-}$ and *Trpc2*$^{-/-}$ mice. VNOx mice had VNO surgical ablation. Responses to female intruders by *Trpc2*$^{-/-}$ and VNOx females was indistinguishable from control and mutant males while responses to intruder males was similar to that of mutant males (error bars are s.e.m; $^*P < 0.05$; $^{**}P < 0.01$, one-way ANOVA followed by post-hoc Tukey Honest tests), suggesting a role of the VNO in sex discrimination in both males and females, and in repressing male-like behaviour in females. NA, not applicable.

Thus, the behaviour of *Trpc2*$^{-/-}$ females highly resembles that typically exhibited by wild-type males interacting with females. Remarkably, in clear contrast with the normal, though rare occurrences of female mounting described in rodents[15–17], the mounting behaviour of the *Trpc2*$^{-/-}$ females was not influenced by their oestrous stage (not shown), and rather than disappearing, it was maintained after sexual experience (Supplementary Fig. 2), and was not associated with dominance and aggression (see arena observation below). Moreover, some male-like behaviours shown by *Trpc2*$^{-/-}$ females, such as pelvic thrusts and sexual solicitation with the snout (Supplementary Videos 1 and 2), are very rarely displayed by normal females. These observations strongly argue that the behaviour of *Trpc2*$^{-/-}$ females does not simply result from a female hypersexual state, but represents genuine abnormal male-like traits that are quantitatively and qualitatively different from normal female behaviour.

Surgical ablation of the VNO in adults

Because the *Trpc2* mutation eliminates VNO function throughout life, we reasoned that the male-like sexual behaviour of *Trpc2*$^{-/-}$ females may result from the abnormal development of female behaviour circuits. Alternatively, it may reveal the de-repression of an existing male behaviour circuit that is normally masked in the female brain by inhibitory vomeronasal inputs. To distinguish between these hypotheses, we compared the behaviour of mice in which the VNO has been surgically removed in the adult to the behaviour of genetically deficient animals of the same age.

Adult olfactory marker protein (OMP)-*TLZ*$^{+/-}$ males and females, in which olfactory expression of tau-LacZ permits direct visualization of main olfactory epithelium and VNO projections[23], had their VNO removed (VNOx) and their airways cleared daily for a week after surgery. Behavioural tests were performed three weeks after surgery, and animals were killed to assess the extent of VNO removal and accessibility of nasal airways (Fig. 3). These additional steps were made necessary by our observation that VNO ablation may cause bleeding and obstruction of the nasal cavity (Supplementary Fig. 3), resulting in behaviour patterns similar to that of olfactory-deficient mice, such as severe deficits in sexual and aggressive behaviour. We also performed VNO surgical ablation in *Trpc2*$^{-/-}$ mice, and showed that mutant animals with or without intact VNOs of a given gender ($n = 9$ each gender) exhibited identical behaviours (not shown), ensuring that no remnants of VNO function can be identified in *Trpc2*$^{-/-}$ animals.

Our data showed that the sexual and courtship behaviours of VNOx males towards male and female intruders are indistinguishable from those of *Trpc2*$^{-/-}$ mutants with intact VNOs. (Fig. 1, Fig. 2 and Supplementary Fig. 1, right side of each histogram). Further, *Trpc2*$^{+/+}$ and *Trpc2*$^{+/-}$ VNOx females exhibited most male-like traits at levels similar to that of *Trpc2*$^{-/-}$ females. These included mounting, pelvic thrust, latency to mount, ultrasound vocalization and olfactory investigation (Supplementary Video 3). We obtained similar results when the VNO removal was performed on C57BL/6J adults (Supplementary Fig. 4), in direct contrast with previously published studies[24,25]. Because these earlier studies did not control for the possible occlusion of the nasal cavity (a frequent occurrence after standard VNO surgical removal), it is possible that additional olfactory deficits have confounded the interpretation of the data.

Thus, the loss of VNO function in adulthood resulted in altered sexual behaviour in males and in sudden sex-reversal of female behaviour, demonstrating the requirement for sustained control by VNO inputs to ensure normal sex discrimination in males and females, and female-specific sexual behaviour. In addition, slight differences in the behaviour of *Trpc2*$^{-/-}$ and VNOx females suggest that VNO activity plays a minor role during development.

Behaviour under semi-natural conditions

When studying complex and dynamic behaviours, such as social interactions, confined experimental conditions could cause the

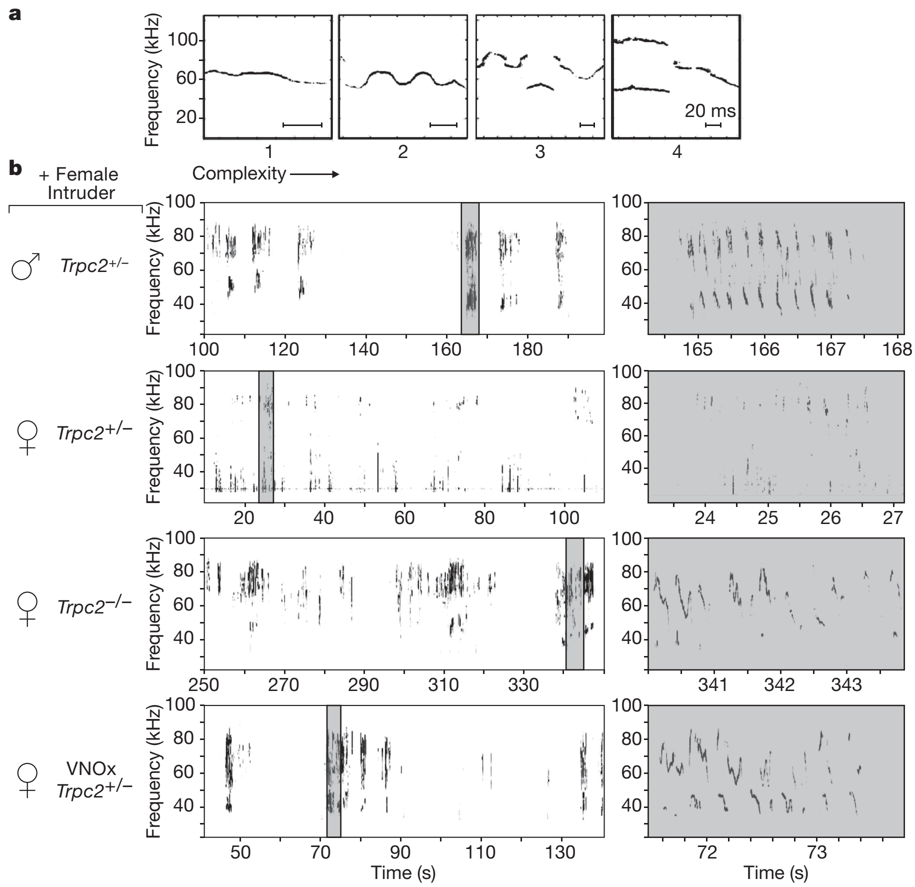

Figure 2 | Ultrasonic vocalization by male and female mice in resident-intruder assays. **a**, Ultrasonic complexity index. Whistle clusters with no pitch jump and temporal overlap in frequency received a low complexity score (1–2), while clusters of whistles with pitch jump and extensive temporal overlap received a high complexity score (3–4). **b**, Representative examples of ultrasonic vocalizations emitted by a *Trpc2*$^{+/-}$ male and female, a *Trpc2*$^{-/-}$ female and a VNOx *Trpc2*$^{+/-}$ female in the presence of a female intruder. Except for the *Trpc2*$^{+/-}$ female, other examples show whistle clusters with highest complexity score (3–4). Power below 35 kHz was truncated.

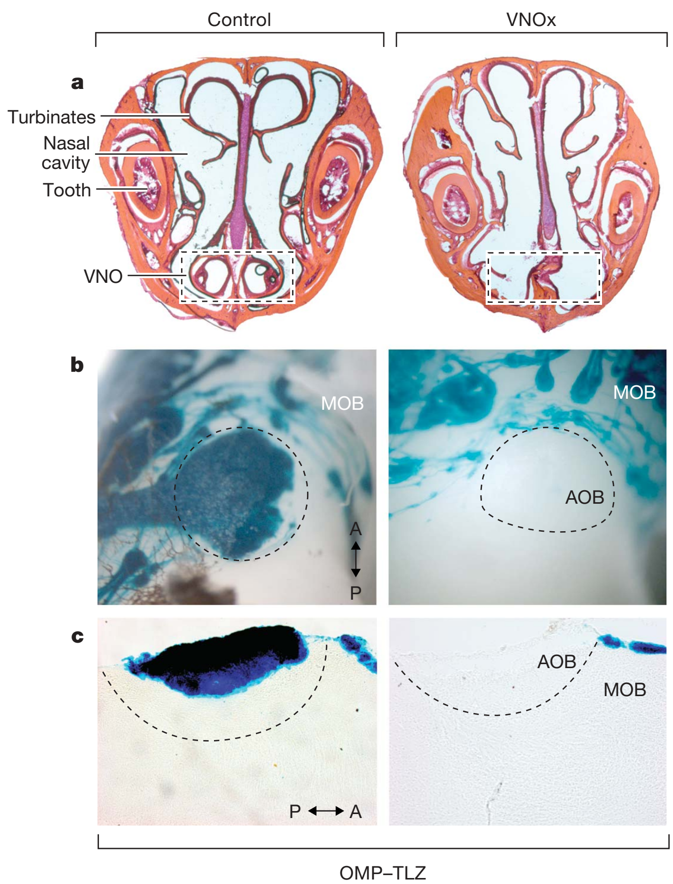

Figure 3 | Surgery leads to a complete removal of the vomeronasal organ (VNOx) while the nasal airways stay clear. **a**, Coronal sections (50 μm) through the anterior part of the skull of control (left) and VNOx mice (right) were stained with haematoxylin and eosin, showing full removal of the bilateral VNO structure while the nasal airway is kept open. **b**, **c**, X-Gal staining of the olfactory bulb of OMP-ires-tauLacZ (OMP-TLZ) control (left) and VNOx mice. **b**, Whole-mount staining. Dorsal view. **c**, Parasagittal sections (50 μm) served as controls for the complete disappearance of all VNO projections to the accessory olfactory bulb. A, anterior; P, posterior; AOB, accessory olfactory bulb; MOB, main olfactory bulb.

animal to present a limited and even impaired behavioural repertoire. Therefore, we also decided to test the role of VNO-mediated pheromone detection in female mice under semi-natural conditions.

Groups of four *Trpc2*$^{+/-}$ and *Trpc2*$^{-/-}$ females were first kept separated in each half of a large enclosure with enriched environment, and were scored for social behaviours. In contrast to the *Trpc2*$^{+/-}$ females, the *Trpc2*$^{-/-}$ females exhibited high levels of social interactions such as mounting, anogenital olfactory investigation, as well as defensive behaviour resulting from mounting attempts by other females (Fig. 4, stage 1). Importantly, neither the *Trpc2*$^{-/-}$ nor the *Trpc2*$^{+/-}$ group established any recognizable dominant–subordinate social hierarchy.

Sexually experienced wild-type males were then introduced into each side of the enclosure. Remarkably, unlike *Trpc2*$^{+/-}$ females (Supplementary Video 4), *Trpc2*$^{-/-}$ females (Supplementary Video 5) intensely chased the males in attempt to investigate their anogenital region and to mount (Fig. 4, stage 2). Again, we found no dominant–subordinate social hierarchy established in either group, and no correlation between the level of female–male mounting in *Trpc2*$^{-/-}$ individuals and the level of aggression towards each other or towards males. Thus, in contrast with normally occurring female–female mounting, the mounting behaviour of *Trpc2*$^{-/-}$ females is unrelated to dominance. Moreover, as shown above, it is not a sexual solicitation of non-responsive males by oestrous females as described in rats[15,16], and instead has characteristics of genuine male sexual behaviour.

Most females within the arena became pregnant and 15 to 22 pups were delivered per group. The successful mating of males with $Trpc2^{-/-}$ females was probably due to increased aggressive behaviour and mating attempts of the males with the $Trpc2^{-/-}$ females. We subsequently tested maternal aggression of lactating females towards intruder males by adding a strange male from the CD1 strain to the enclosure. All lactating $Trpc2^{+/-}$ females attacked the intruder male and showed low sexual receptivity. In contrast, the intruder male evoked a low level of aggression from the $Trpc2^{-/-}$ females, which appeared highly sexually receptive (Fig. 4, stage 3). Our findings confirm previous results showing low aggression from lactating $Trpc2^{-/-}$ females[10] while contradicting the described deficiency in sexual receptivity of VNOx females[24]

Finally, we investigated female maternal and lactating behaviours. Both $Trpc2^{+/-}$ and $Trpc2^{-/-}$ females kept their litters in one common breeding nest where the females nursed the pups in turn. In the first and second days after birth, both groups spent a similar amount of time with their pups. However, during the following days, $Trpc2^{-/-}$ females exhibited a significant decrease in time spent in the breeding nest, while $Trpc2^{+/-}$ females spent most of their time in the breeding nest. On the last day (day 14), the partition between the $Trpc2^{-/-}$ and $Trpc2^{+/-}$ females was lifted to allow both groups to interact. Strikingly, the $Trpc2^{-/-}$ females immediately abandoned the breeding nest to explore the $Trpc2^{+/-}$ territory, while $Trpc2^{+/-}$ females remained in breeding nests and continued to nurse their pups (Fig. 4, stage 4). These findings indicate that $Trpc2^{-/-}$ females display a deficiency in maternal behaviour.

Discussion

We have shown here that $Trpc2^{-/-}$ females exhibit robust male-like sexual and courtship behaviours and display a reduction in female-specific behaviours such as maternal aggression and nesting. These findings suggest that VNO-mediated inputs repress male-like sexual and courtship behaviours in females, and activate and sustain female maternal behaviours.

Previous studies have suggested a central role for sex hormones in the regulation of male- and female-specific behaviours[26]. We found that body weight and oestrous cycles of $Trpc2^{-/-}$ females (Fig. 5a) appeared normal. Moreover, radioimmunoassay for testosterone and 17β-estradiol in the serum of $Trpc2^{-/-}$ and $Trpc2^{+/-}$ males and females reveal that sex steroid levels remain within the normal range for each gender (Fig. 5a). A minor increase in free testosterone level was observed in $Trpc2^{-/-}$ females that still vastly differs from typical male levels and from the amounts of exogenous testosterone

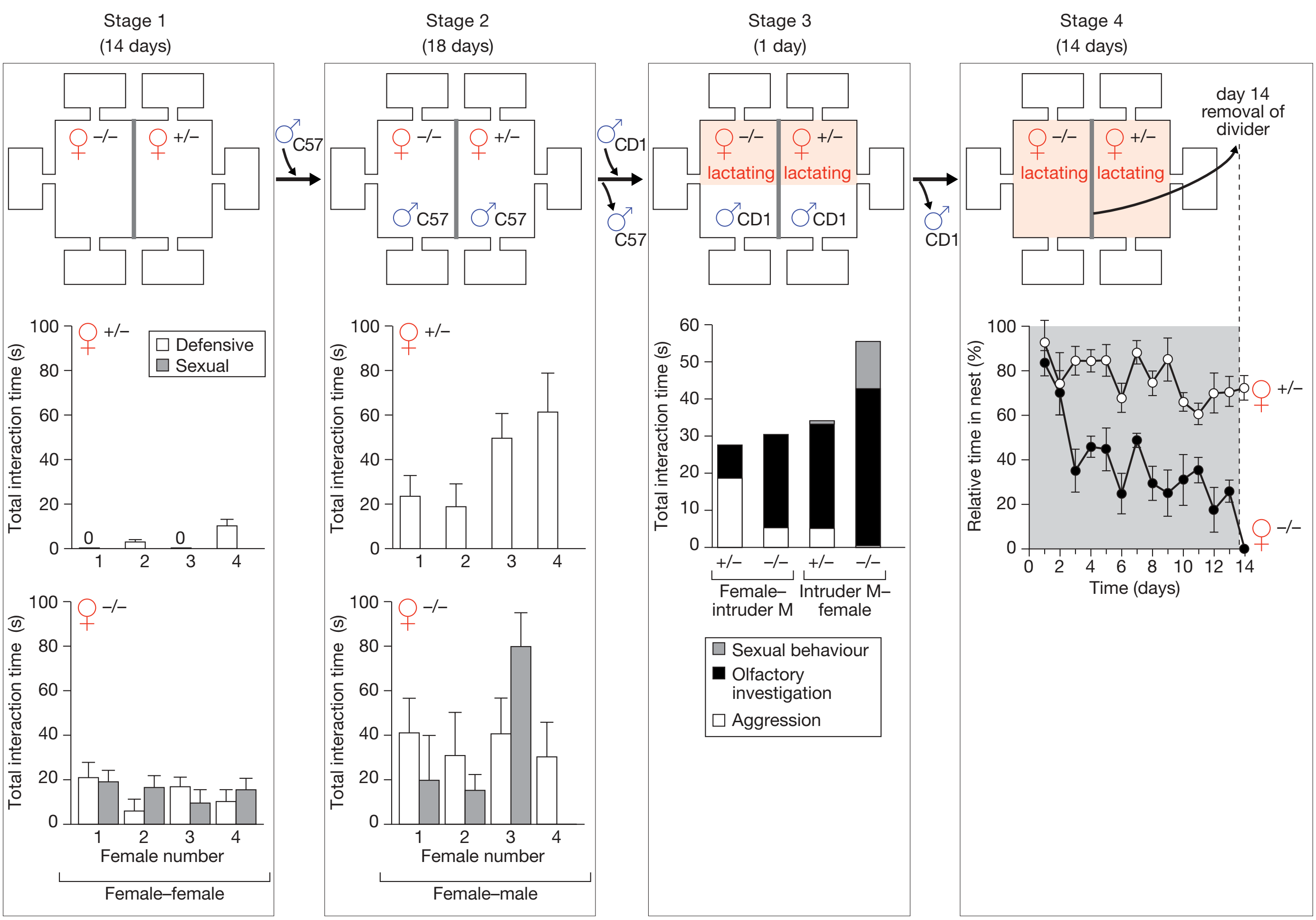

Figure 4 | Social behaviour of $Trpc2^{+/-}$ and $Trpc2^{-/-}$ females in semi-natural conditions. Stages 1 and 2 show duration of defensive (white) and sexual (grey) behaviours of resident females with each other (stage 1) and towards a C57BL/6J adult male (stage 2). Stage 3 shows duration of sexual behaviour (grey), olfactory investigation (black) and aggression (white) of the resident lactating females towards an intruder CD1 adult male (left two columns) and behaviour of intruder CD1 towards the resident females (right two columns). Stage 4 shows relative time spent by lactating $Trpc2^{+/-}$ and $Trpc2^{-/-}$ females in the breeding nest before and after (day 14) the divider was lifted. In the enriched environment $Trpc2^{-/-}$ females exhibit high levels of male-like sexual behaviour (stages 1 to 2). They exhibit no apparent deficiency in sexual receptivity but show reduction in maternal (stage 3) and lactating behaviour (stage 4). Stages 1–4, error bars are s.e.m.

required to affect normal female behaviour[27,28] Thus, hormonal changes do not seem to underlie the expression of male-like behaviours in *Trpc2*$^{-/-}$ females.

The prevailing model for the sexual dimorphism of behaviours is that the sex hormone testosterone initiates the development of male-specific circuitry in the central nervous system and the activation of male-specific neuronal networks in adulthood[26]. However, our results clearly reveal that a functional neuronal network mediating male sexual behaviour develops and persists in females. These findings suggest a new model of sexual dimorphism in which the effector circuits of both male and female behaviours exist in the brain of each sex, and are switched on or off by gender-specific sensory modulators (Fig. 5b). Interestingly, one or a few classes of olfactory receptors have been shown in *Drosophila* to mediate the specificity of male and female sexual responses in a similar way[29,30]. Further support for our model in the mouse will involve the identification of specific receptor inputs that inhibit the expression of male responses in the female brain. Moreover, a similar inhibitory control of female behaviour may yet be found in the male brain.

a

	Trpc2$^{+/-}$	*Trpc2*$^{-/-}$	*P* value
Weight of animals (g)			
Males (*n* = 15)	25.67 ± 0.66	25.96 ± 0.68	NS
Females (*n* = 26)	21.41 ± 0.69	22.43 ± 0.56	NS
Duration of oestrous cycle (days)			
Females (*n* = 10)	5.20 ± 0.25	5.40 ± 0.26	NS
Steroid hormone level in blood			
Total testosterone (ng ml^{-1})			
Males (*n* = 5)	3.10 ± 0.30	4.50 ± 0.60	NS
Females (n=6)	<0.1	<0.1	
Free testosterone (pg ml^{-1})			
Males (*n* = 5)	14.10 ± 1.30	15.30 ± 1.50	NS
Females (*n* = 6)	0.19 ± 0.07	0.41 ± 0.03	<0.05
17β estradiol (pg ml^{-1})			
Males (*n* = 5)	21.31 ± 3.20	20.58 ± 2.80	NS
Females (*n* = 6)	14.29 ± 2.10	14.91 ± 1.50	NS

b

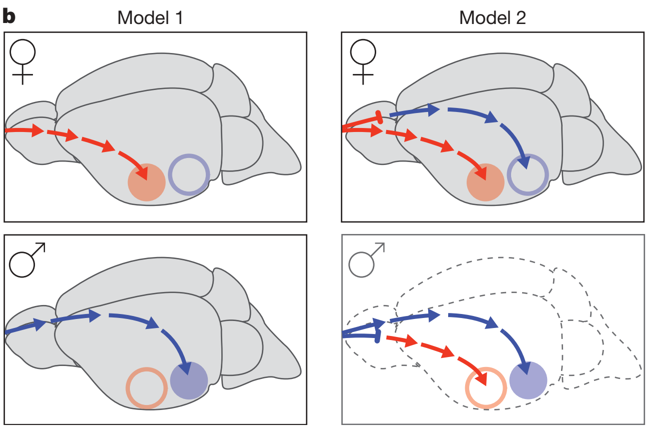

Figure 5 | Sexual dimorphic traits in *Trpc2*$^{-/-}$ mutants and regulation of sexually dimorphic behaviour. a, *Trpc2*$^{-/-}$ female body weight, oestrous cycle and steroid hormone levels are in the range of that of heterozygote females. Values are means ± s.e.m. NS = $P > 0.1$, Student's *t*-tests for independent samples. **b**, Prevailing views on neuronal networks underlying sexual dimorphic behaviours (model 1) have assumed the existence of sex-specific circuits throughout the brain, including male (blue) and female (red) specific sensory and effector networks. In contrast, data resulting from VNO deficiency in females support a different model (model 2), according to which effector networks for sex-specific behaviours are present in both females (our data) and males (dotted lines, to be determined), while the sensory switch is sexually dimorphic.

METHODS SUMMARY

Behavioural assays. We used sexually naive, 2–3-month-old *Trpc2*$^{+/+}$, *Trpc2*$^{+/-}$, *Trpc2*$^{-/-}$ (ref. 9), and C57BL/6J × 129/Sv mice of mixed genetic background as well as C57BL/6J mice in the behaviour experiments. Mice were tested in 15 min intruder–resident assays in which individually housed residents mice were exposed to sexually naive female, and either castrated or olfactory bulboctemized male C57BL/6J intruders. Ultrasound vocalizations by the resident mice were recorded during intruder–resident assays. Stored recordings were processed using a custom MATLAB-based program as described[18]. Social behaviour within a colony of *Trpc2*$^{+/-}$ and *Trpc2*$^{-/-}$ mice was recorded under semi-natural conditions within a large (120 cm × 90 cm × 80 cm), environmentally enriched enclosure. The behaviour of the mice was recorded by low-light-sensitive video cameras that were connected to a custom-designed PC-based recording unit (Protech PC). The social behaviour (courtship and sexual behaviours) in both the intruder–resident assays and in the semi-natural conditions were scored in the recorded videos using Observer Video Pro software (Noldus).
Surgical VNO removal. The VNO was surgically ablated from adult, 8–10-week-old, sexually naive mice (*Trpc2*$^{+/-}$ × OMP-tauLacZ and *Trpc2*$^{-/-}$ × OMP-tauLacZ). For the first week after surgery, VNOx mice were anaesthetized daily, their nostrils were rinsed with 0.9% saline solution, and any blood clots were gently aspirated. VNOx mice were allowed at least three weeks to recover before behavioural testing.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Intruder–resident assay. *Trpc2*$^{+/-}$ and *Trpc2*$^{-/-}$ (ref. 9) sexually naive, 2–3-month-old, female and male littermates of the C57BL/6J × 129/Sv mixed genetic background were housed individually in a cage for one to two weeks before the beginning of the experiment. Experiments started at the beginning of the dark phase and were performed under dim red light.

The following two kinds of intruders (C57BL/6J) were introduced to the resident mouse cage: sexually naive, receptive female (as determined by vaginal smear), 7–8 weeks old; and sexually naive, castrated or olfactory bulboctemized male, 6–7 weeks old, swabbed with urine from intact wild-type males. Each 15 min assay was videotaped and scored for the following three behaviours: sexual behaviour (mounting, and mounting with pelvic thrust); solicitation behaviour (resident animal lifts intruder's rear with its snout); and olfactory investigation.

Recording and processing of ultrasonic vocalization. Sounds over the frequency range of 20 Hz–110 kHz were recorded with a microphone and amplifier (Bruel & Kjaer) and digitized at 250 kHz, 16 bits (National Instruments) and saved to disk within a custom-designed MATLAB-based program.

In brief, the MATLAB-based program converted the stored waveforms to sonograms (512 sample/blockform, time resolution of 1.02 ms and a frequency of 0.98 kHz), removing white noise outside the range 25–110 kHz; it identified and presented each ultrasonic mouse whistle. The MATLAB-based program was used to estimate the whistling activity level by summing the overall whistling time out of the total whistling clusters time for each observation. Whistle clusters were classified by the criteria that there existed at least five whistles where the period of time between each whistle was less than 500 ms, and additionally, that the beginning and end whistle were separated from the previous and next whistles, respectively, by at least 500 ms.

To confirm that the resident mouse was the source of the ultrasonic vocalizations we recorded from assays in which either the resident or the intruder mouse was anaesthetized. We were only able to record robust ultrasonic vocalizations resembling those we recorded during the resident–intruder assays if the intruder mouse was anaesthetized and not the resident.

Semi-natural experimental set-up and procedure. Sexually naive, 3–4-month-old females ($n = 4$ each genotype) *Trpc2*$^{-/-}$ and *Trpc2*$^{+/-}$ mice, derived from four different litters, were used. The female mice were housed individually in a cage two weeks before the beginning of the test. To enable us to individually recognize the animals, before the beginning of the experiment the animals were anaesthetized (with 120 mg of ketamine per kg of mouse body weight and 10 mg kg^{-1} xylazine) and marked with commercial hair dye. The mice were re-marked every two to three weeks throughout the experiment. The experiment was conducted twice with different animals.

Semi-natural enclosure set-up. The enclosure consisted of a large central arena constructed from transparent polycarbonate boards that were connected to six peripheral standard mouse cages by short transparent tubes. A removable oblique polycarbonate board (90 × 80 cm) served as a divider. The floor of the arena and cages were spread with bedding, scattered strips of towel paper, cotton pieces, and shelter boxes. In addition, each half of the arena contained a central platform with rodent pellets and water freely supplied.

The enclosure was placed in a temperature- and light-controlled (12 h:12 h light/dark cycle) room furnished with infrared lights and equipped with low-light-sensitive cameras mounted above and around the enclosure. All cameras were connected to a digital video recording unit (ProtechPC).

The behaviour was recorded daily for 10–20 min every hour, from the beginning of the dark phase until 2 h before the light phase. The data were scored using the Observer Video Pro software (Noldus).

Testosterone and oestrogen blood level measurements. Animals were killed by overdose of 2.5% avertin. Blood was removed from the heart, and serum or plasma was separated and stored at −20 °C until analysed. Concentrations of testosterone and 17β-estradiol were measured using a radioimmunoassay RIA Kit (MP Biomedicals).

Surgical VNO removal. Mice were anaesthetized (120 mg kg^{-1} ketamine and 10 mg kg^{-1} xylazine), placed supine in a head holder, and the lower jaw was gently opened. A midline incision was made in the soft palate, extending rostrally from behind the first palatal ridge to the incisors, and the underlying bone was exposed. The caudal end of the vomer bone was cut and the VNO was removed bilaterally. Low-pressure vacuum was used to clear blood from the mouse and nostrils during the surgery. The VNO cavity was packed with absorbable gel foam (Pharmacia) and the incision was closed with veterinary sterile tissue adhesive (Tissumend II).

After testing, VNOx mice were killed to confirm complete VNO removal. To confirm complete degeneration of axonal projections to the accessory olfactory bulb, we performed whole-mount X-Gal staining of the olfactory bulbs, as previously described[23], followed by 50 μm parasagittal sections of the stained tissue, for more detailed examination. To confirm that there were no blood clots blocking the olfactory airways, skulls of VNOx mice were decalcified in 10% EDTA (pH = 7.4), sectioned coronally (50 μm) on a cryostat, counterstained with haematoxylin and eosin, and dehydrated.