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Deficient pheromone responses in mice lacking a cluster of vomeronasal receptor genes

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The mammalian vomeronasal organ (VNO), a part of the olfactory system, detects pheromones—chemical signals that modulate social and reproductive behaviours[1,2]. But the molecular receptors in the VNO that detect these chemosensory stimuli remain undefined. Candidate pheromone receptors are encoded by two distinct and complex superfamilies of genes, *V1r* and *V2r* (refs 3 and 4), which code for receptors with seven transmembrane domains. These genes are selectively expressed in sensory neurons of the VNO. However, there is at present no functional evidence for a role of these genes in pheromone responses. Here, using chromosome engineering technology[5], we delete in the germ line of mice a ~600-kilobase genomic region that contains a cluster of 16 intact *V1r* genes[6]. These genes comprise two of the 12 described *V1r* gene families[7], and represent ~12% of the *V1r* repertoire. The mutant mice display deficits in a subset of VNO-dependent behaviours: the expression of male sexual behaviour and maternal aggression is substantially altered. Electrophysiologically, the epithelium of the VNO of such mice does not respond detectably to specific pheromonal ligands. The behavioural impairment and chemosensory deficit support a role of V1r receptors as pheromone receptors.

Vomeronasal sensory neurons (VSNs) in the mammalian VNO are thought to be specialized in the detection of pheromones[8,9], although their chemoreceptive abilities also extend to other types of ligands[10]. Much of our knowledge about mammalian VNO function has been obtained by describing the behavioural consequences of surgical lesions. Animals with complete anatomical removal of the VNO exhibit a range of deficits in social and reproductive behaviours, including the emission of male ultrasound vocalizations to females, intermale aggression, male sexual behaviour, and maternal aggression[11]. Pheromones, present in bodily secretions such as urine[1,2], elicit these VNO-dependent behaviours. *V1r* (ref. 3) and *V2r* (ref. 4) genes are proposed to encode candidate chemosensory receptors, specifically pheromone receptors, on the basis of predicted protein structure, patterns of expression, and genetic complexity. However, no ligands, natural or synthetic, are known for specific V1r or V2r receptors and there is no functional evidence implicating these receptors in pheromonal behaviours.

To determine if V1r receptors are involved in behavioural and electrophysiological responses to pheromones, we deleted a cluster of *V1r* genes in the germ line of mice. We[6] and others[12] have characterized the genomic organization of a cluster of *V1r* genes on chromosome 6. A ~600-kilobase (kb) region comprises 23 *V1r* genes of which 16 have an intact open reading frame (ORF); the remaining 7 *V1r* genes are pseudogenes, as their ORFs are disrupted. The mouse *V1r* superfamily[7] consists of ≥137 genes with an intact ORF that can be grouped into 12 phylogenetically highly isolated families (*V1ra-l*) (Fig. 1a). Our cluster represents ~12% of the potentially functional V1r repertoire, and contains most members of two *V1r* families, *V1ra* and *V1rb* (Fig. 1a, b), but no other genes[6,12]. To delete the gene cluster we used the technique of chromosome engineering[5], designed to excise large regions of genomic DNA via gene targeting and Cre-*loxP* mediated site-specific recombination in embryonic stem cells (Fig. 1b). The resulting homozygous mice, termed *ΔV1rabΔ* mice, harbour a ~600-kb genomic deletion and lack most *V1ra* and *V1rb* genes, as evidenced by Southern blot hybridization with a *V1ra/b* probe (Fig. 1c). One intact family member, *V1rb10*, located on the X chromosome[6], is not included in the deletion. *In situ* hybridization of the vomeronasal epithelium confirmed the expected absence of messenger RNA for the deleted *V1r* genes in the mutant mice (Fig. 1d). The expression of other families of *V1r* genes and the thickness of the layer of *V1r*-expressing VSNs are not obviously affected (Fig. 1d). The accessory olfactory bulb (AOB), which receives axonal input from VSNs, shows a normal overall size, layered organization, and rostral segregation of axons from VSNs expressing $G\alpha_{i2}$ (Fig. 1e, f).

Table 1 **VNO-independent functions**

	Genotype	
	WT	−/−
Weight		
Males (15-20 weeks) (g)	19.9 ± 3.3	19.0 ± 3.6
Females (17-19 weeks) (g)	16.0 ± 4.3	16.7 ± 4.4
Motor activity: bar hanging and balancing		
Time spent hanging on wire (s)	39.1 ± 7.6	41.9 ± 6.1
Time to right on wire (s)	2.8 ± 0.6	2.4 ± 0.5
Motor activity: screen climbing		
Time to climb to top of screen (s)	24.6 ± 2.8	26.7 ± 2.5
Locomotor activity in open field		
Total moving distance males (cm)	470.9 ± 93.2	456.8 ± 84.1
Total moving distance females (cm)	370.4 ± 85.6	422.2 ± 71.9
Depression-related behaviours		
Forced swim test (total immobility: min)	92.2 ± 14.4	84.3 ± 11.7
Tail suspension test (total immobility: min)	107.1 ± 5.6	109.2 ± 7.9
Oestrous cycling of females		
Mean number of cycles in 17 d	1.7 ± 0.1	1.8 ± 0.2
Olfactory function: latency to find cookie		
Day 1 (s)	218.3 ± 25.3	179.2 ± 25.9
Day 2 (s)	114.4 ± 20.6	89.9 ± 17.4
Day 3 (s)	86.0 ± 9.5	87.0 ± 10.3
Day 4 (s)	56.4 ± 6.4	58.3 ± 9.3

Body weight of males and females ($n = 30$ per genotype and sex), motor activity ($n = 20$ per genotype), locomotor activity ($n = 20$ per genotype and sex), depression-related behaviours ($n = 15$ per genotype and per test) and the number of oestrous cycles of non-group-housed females ($n = 15$ per genotype) were not significantly different between wild-type (WT) and mutant (−/−) mice as evaluated with an unpaired Student's *t*-test. The test of olfactory function was repeated over four consecutive days. Both genotypes learn to find the cookie faster with repeated trials. No significant differences were observed between the genotypes ($n = 15$ per genotype and sex), as evaluated with a two-way ANOVA for repeated measurements for main effects of genotype and test day and their interaction.

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The *ΔV1rabΔ* strain was established and analysed in an inbred 129/SvEv background. The mutant mice are viable, fertile and overtly normal. Gross body weight throughout development, motor activity, open-field locomotor activity, depression-related behaviours, and oestrous cycling of females are indistinguishable between mutant and wild-type mice (Table 1). Mutant mice do not show deficits in a food-finding test, a global measure of function of the main olfactory system (Table 1). Thus, *ΔV1rabΔ* mice do not display dysfunctions that would complicate the interpretation of their performance in VNO-dependent behavioural tests.

Maternal aggression is strongly dependent on a functional VNO[13]. Normally, lactating females are aggressive towards intruders invading their nest area; however, all measures of aggression are significantly reduced in nursing *ΔV1rabΔ* females: the latency to the first attack, the total time attacking, the number of attacks, the number of fights, and the number of tail rattles (Fig. 2a). The deficits are more pronounced immediately after exposure to the intruder (Fig. 2b) and attenuate as the test proceeds. Thus, the deleted *V1r* genes are involved in maternal aggression towards an intruder mouse, particularly in the acute onset of the behaviour. Other aspects of maternal behaviour are normal: the average time spent retrieving pups back to the nest is not significantly different between the genotypes (Fig. 2c), in accord with normal pup retrieval after VNO removal[11].

We assayed four behaviours in males. A first test focused on the 70-kHz ultrasound vocalizations emitted by males during the first minutes of exposure to a female mouse. Although this behaviour is attenuated when the VNO is removed[14,15], it is not altered in the mutant mice (Fig. 3a). Second, we assayed mutant males for their display of aggressive behaviours towards other, wild-type males. This VNO-dependent behaviour[16,17] is not affected in the mutant mice (Fig. 3b): the percentage of aggressive mutant mice and their level of aggression (data not shown) are not significantly different from wild-type mice. Third, male–male sexual behaviour was scored during the aggression tests. It is commonly observed that socially inexperienced, wild-type mice exhibit sexual behaviour (composed of mounting and rhythmic, vigorous pelvic thrusting) towards other males. The expression of this behaviour diminishes as the mice learn to discriminate between sexes through social experience (Fig. 3c)[18]. Interestingly, for socially inexperienced mice (test 1) the level of male–male sexual behaviour is significantly lower in mutant compared to wild-type mice, and is comparable to that displayed by wild-type mice after social experience (tests 2 and 3). Thus, mutant males can discriminate better between sexes without prior experience, or their sexual drive is globally diminished.

A fourth assay of the mutant males tested their sexual behaviour towards females, which is also VNO-dependent[11,17]. The percentage of males that mounted the female was lower in mutant compared to wild-type mice, in each of five consecutive tests (Fig. 3d). The effect tends to become stronger with consecutive trials. Normally the

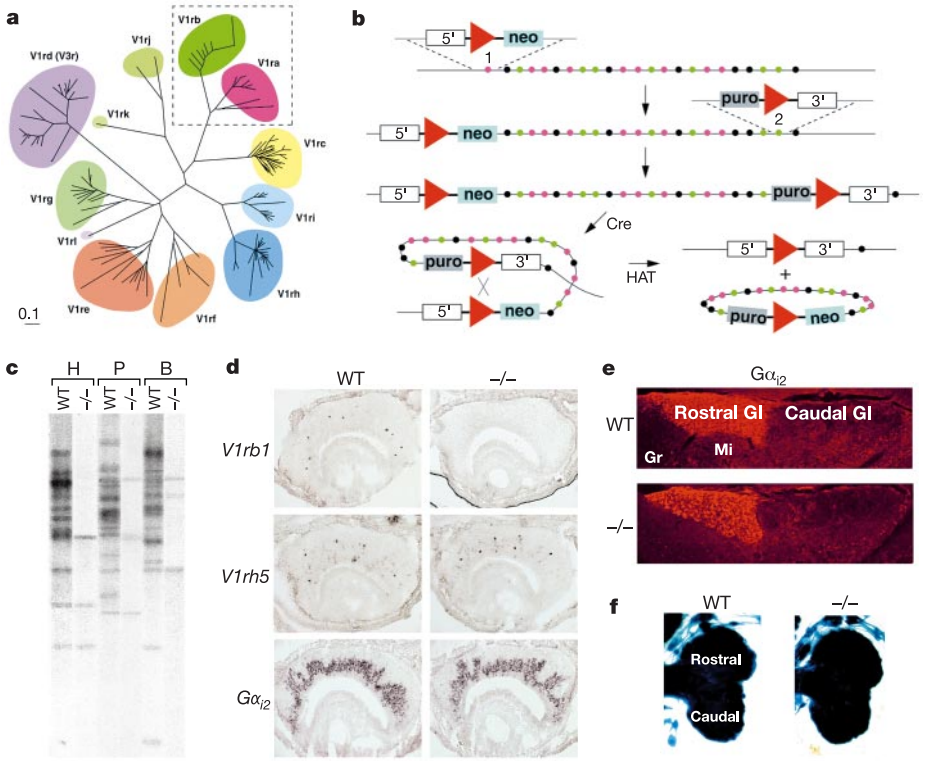

Figure 1 Targeted deletion of a cluster of *V1r* genes. **a**, The mouse V1r repertoire[7]. Dashed lines enclose V1ra and V1rb families. **b**, Gene targeting strategy. Top horizontal line represents the cluster[6,12] containing *V1ra* (pink dots) and *V1rb* (green) genes. From left to right: *V1ra9*, *V1rb3*, *V1ra8*, *V1ra7*, *V1ra1*, *V1rb1*, *V1rb2*, *V1ra4*, *V1ra3*, *V1rb8*, *V1ra2*, *V1rb4*, *V1ra5*, *V1ra6*, *V1rb9*, *V1rb7*. Black dots, pseudogenes. Replacement vectors, containing the selectable marker genes *neo* or *puro*, a *loxP* site, and exons 1–2 (5′) and 3–9 (3′), respectively, of the *Hprt* gene, were consecutively targeted (1 and 2) to the *V1ra9* and *V1rb7* loci in ES cells. Cre-mediated recombination between *loxP* sites and HAT selection gave cells with a reconstituted *Hprt* gene, and thus a deletion of the cluster, lost as circular DNA. The deletion encompasses all *V1ra/b* genes with an intact ORF in the cluster, from *V1ra9* to *V1rb7* inclusive. **c**, Southern blot of genomic DNA with a *V1ra/b* probe[6]. WT, wild type; −/−, *ΔV1rabΔ* mice; H, *Hin*dIII; P, *Pst*I; B, *Bam*HI. **d**, *In situ* hybridization with a probe of a *V1r* gene residing within the cluster (*V1rb1*, top), a *V1r* gene outside the cluster (*V1rh5*, middle), and a $G\alpha_{i2}$ probe, which labels *V1r*-expressing neurons (lower). **e**, Immunostaining of an AOB sagittal section with an anti-$G\alpha_{i2}$ antibody, which labels axons of *V1r*-expressing neurons projecting to the rostral region. Gl, glomerular layer; Mi, mitral cell layer; Gr, granule cell layer. **f**, Whole-mount dorsal view of the AOB of an *OMP-taulacZ* heterozygous mouse[23] (WT) and an *OMP-taulacZ* heterozygous, *ΔV1rabΔ* mouse (−/−) stained with X-gal. In *OMP-taulacZ* mice, all VSNs and their axons are stained blue.

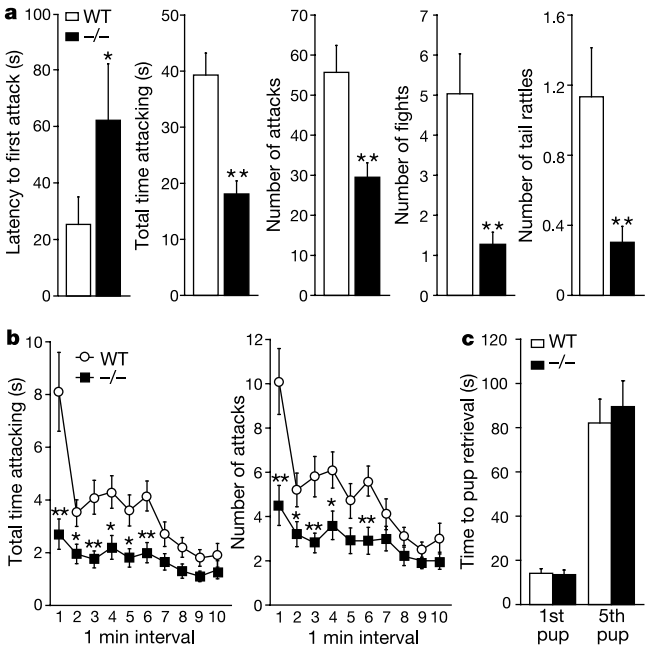

Figure 2 *ΔV1rabΔ* females exhibit a reduced level of maternal aggression. **a**, Maternal aggression of WT versus mutant mice during a 10-min exposure to an unfamiliar male mouse. Data were analysed with an unpaired Student's *t*-test. **b**, Total time attacking (left) and number of attacks (right) during each 1 min interval throughout the test for WT and mutant mice. Data were analysed by a two-way analysis of variance (ANOVA) for repeated measurements for main effects of genotype and test day and their interaction, followed by *post-hoc* one-way ANOVA on each minute. Main effects of genotype, time-point, as well as interaction between these two factors were highly significant ($P < 0.001$, $P < 0.0001$ and $P < 0.002$, respectively). **c**, Performance in the pup retrieval test is not significantly different (Student's *t*-test). Data are means of 34 mice per genotype for **a**, **b** and of 35 for **c**. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$ versus WT mice.

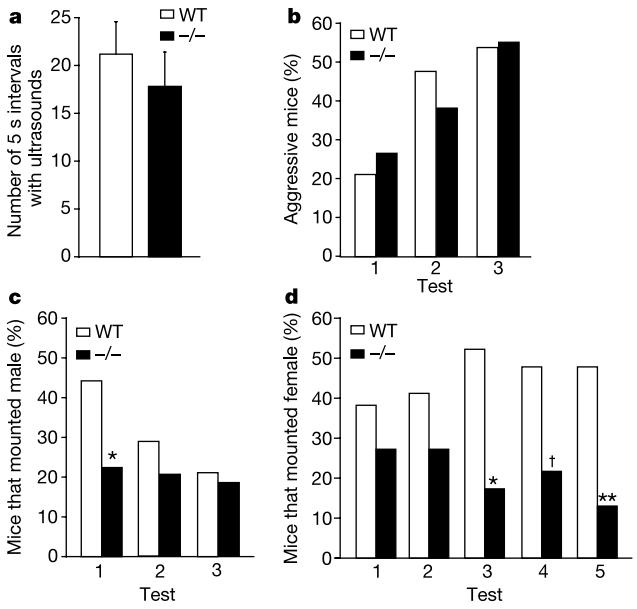

Figure 3 Behaviour of *ΔV1rabΔ* males. **a**, Ultrasonic vocalizations. Mean number of 5-s intervals containing male ultrasound vocalizations to a female during a 3-min test. Data were analysed with an unpaired Student's *t*-test. $N = 20$ mice per genotype. **b**, Intermale aggression. Percentage of resident WT versus mutant mice that exhibited at least one attack towards an intruder mouse during a resident-intruder aggression test of 15 min performed in three consecutive days. $N = 45$ mice per genotype. **c**, Male–male sexual behaviour. Percentage of male mice that mounted another male at least once during the same tests performed in **b**. $N = 45$ mice, same as in **b**. **d**, Male–female sexual behaviour. Percentage of mice that mounted a receptive female at least once during each of 5 tests of 30 min duration. $N = 35$ WT and 34 mutant mice. Differences in percentages were tested with χ^2. Dagger, $P < 0.07$; asterisk, $P < 0.05$, double asterisk, $P < 0.01$ versus WT mice.

display of sexual behaviour increases with continuous exposure to a female (Fig. 3d). This gain of sexual activity through experience does not occur in the mutant mice—instead, a decreasing tendency is seen (Fig. 3d). The diminution of the response with repetitive trials is in agreement with reports of sexual behaviour in males upon surgical removal of the VNO[17] and is consistent with the proposed view of the VNO as intrinsically rewarding[1,11]. The percentage of mutant mice that ejaculated was half of that of wild-type mice. However, if a mutant male mounted a female during a test, it displayed a level of sexual activity comparable to that of wild type in terms of the latency, number of mounts, and whether or not it ejaculated (data not shown). Thus, although fertile, mutant males have a reduced drive to initiate sexual encounters. Wild-type and mutant mice showed no differences in the levels of plasma testosterone after exposure to female mice (10.5 ± 1.2 versus $9.3 \pm 1.6\,\mathrm{ng\,ml^{-1}}$, $n = 12$ and $n = 11$ wild-type and mutant mice, respectively), ruling out a deficient testosterone response as an explanation.

The behavioural dysfunction of *ΔV1rabΔ* mice may result from peripheral chemosensory deficits caused by the absence of a subset of V1r receptors. To test this hypothesis, we used an intact VNO preparation to record local field potentials (the electro-vomeronasogram, EVG) from the surface of the sensory epithelium[9]. VSNs in the apical layer of mouse VNO exhibit narrow tuning profiles for stimulus discrimination[9]. If V1r receptors function as chemoreceptors in these neurons, deletion of *V1r* genes should cause discrete deficits in the ability of the VNO to detect specific molecules. We compared field potentials in wild-type and mutant mice in response to eight pheromonal ligands known to activate apical VSNs (ref. 9, and T.L.-Z. and F.Z., data not shown) (Fig. 4). VSNs from *ΔV1rabΔ* mice do not respond to 6-hydroxy-6-methyl-3-heptanone, *n*-pentyl acetate and isobutylamine, but are otherwise normal in their responses to the other tested compounds (Fig. 4). Thus, as measured with this technique, deletion of the *V1r* gene cluster abolishes the electrophysiological response to three out of eight ligands tested. We introduce the term 'specific avnosmia' for these specific chemosensory deficits, by analogy with 'specific anosmia' in the main olfactory system[19]. The small proportion of VSNs (<1%) in the apical layer of wild-type mice that respond physiologically to a given pheromone with a calcium increase[9] suggests that a single *V1r* gene may correspond to each specific avnosmia.

Our analyses of *ΔV1rabΔ* mice provide two complementary lines of evidence for a pheromone receptor function of V1r receptors. First, mutant males have a reduced sexual drive and mutant females display a reduced level of maternal aggression. Second, their VNO exhibits specific chemosensory deficits. A chemosensory role of V1r receptors is consistent with immunolocalization of V1r receptors to microvilli of VSNs[20], and can be proved conclusively by functional gene transfer; we have reduced the complexity of this task to three ligands and 16 receptors. An indirect, but not mutually exclusive role is based on the observation that deletion of the *V1r* ORF precludes axonal convergence to glomeruli and results in fewer VSNs expressing the gene[21,22]. This effect is also seen with odorant receptors, which have dual roles both in chemosensation and axon guidance[23]. The impairments that result from the deletion of only ~12% of the functional *V1r* repertoire (or ~5% of the combined *V1r* and *V2r* repertoires) attest to a low level of functional redundancy within the *V1r* repertoire, in contrast to common notions

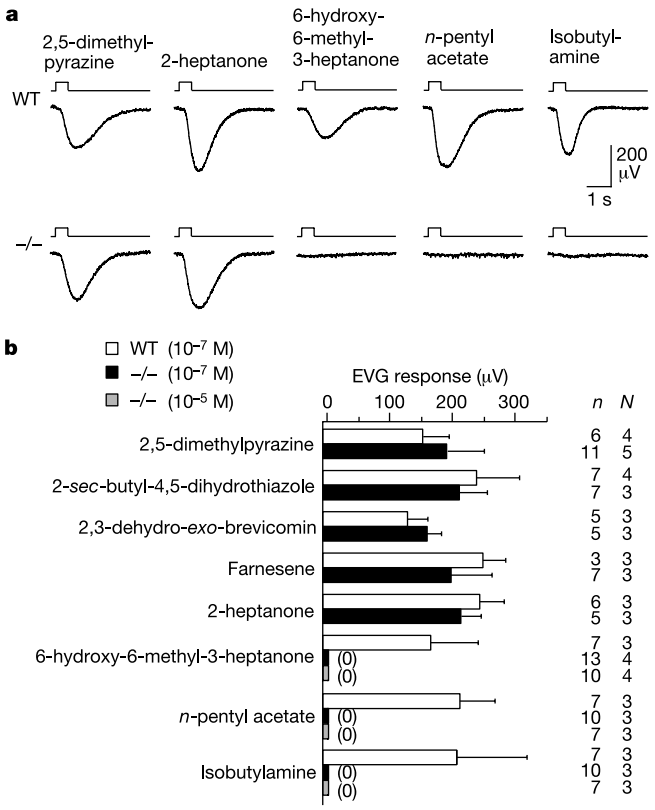

Figure 4 Chemosensory deficits in *ΔV1rabΔ* mice. **a**, Comparison of EVG responses from WT (upper traces) and mutant mice (lower traces). Field potentials were induced by 500-ms pulses of the ligands shown (all at 10^{-7} M). **b**, Histograms showing collected results (mean ± s.d.), based on recordings from 9 WT mice (5 females, 4 males) and 11 mutant mice (5 females, 6 males). Peak EVG responses from both sexes were pooled. Both male and female mutant mice were defective in sensing 6-hydroxy-6-methyl-3-heptanone, *n*-pentyl acetate, and isobutylamine ($P < 0.0001$). There is no significant difference between WT and mutant mice in the detection of the other five tested ligands ($P = 0.1$–0.33). Stimulus concentration, 10^{-7} M (white and black bars) or 10^{-5} M (grey bars). *n*, number of recordings; *N*, number of mice.

about odorant receptors and the main olfactory system[24,25]. Functional specialization is consistent with the phylogenetic isolation of the *V1r* families[7], and with the narrow tuning of VSNs located in the apical layer to single ligands[9]. As pheromones in other species often consist of blends of compounds that must be present in carefully balanced ratios to elicit distinct behaviours[26,27], the deletion of *V1ra* and *V1rb* gene families may produce specific avnosmias to certain critical components, leading to an altered representation of the blend in the brain and affecting behaviour. Deletion of a *V1r* subset may 'corrupt' pheromone coding instead of blocking it, and may thus result in phenotypes that are qualitatively distinct from those produced by VNO removal, a total receptor deletion or a global deficiency in cell signalling, such as reported in Trp2 knockout mice[28,29]. It will be interesting to determine if deletion of other groups of *V1r* or *V2r* genes produces distinct behavioural and electrophysiological phenotypes. Single *Vr* gene knockouts may suffice to cause deficits. □

Methods

Genetic manipulation

Replacement vectors for each end of the *V1r* gene cluster were constructed using the chromosome engineering cassettes *Hprt Δ5′* and *Hprt Δ3′* (ref. 5). The vectors were electroporated consecutively into AB2.2 embryonic stem cells[30], derived from an Hprt-deficient 129/SvEv blastocyst. Clones with the double mutation were electroporated with the Cre-expression plasmid pOG231. The vectors were constructed such that recombination between the *loxP* sites reconstitutes a functional *Hprt* gene. Cells that had undergone recombination and thus expressed Hprt were selected in hypoxantine-aminopterin-thymidine (HAT) medium. Sib selection analysis showed that HAT-resistant clones had regained sensitivity to neomycin and puromycin, indicating that a recombination *in cis* had occurred and confirming the deletion event. Male chimaeras were crossed to 129/SvEv females (Taconic) to maintain an inbred genetic background more suitable and reproducible for the behavioural experiments. As controls, we used wild-type offspring from heterozygous intercrosses, or wild-type offspring from wild-type parents specifically bred for this purpose. Offspring lacking the mutation were bred in parallel to produce wild-type mice. The *V1ra/b* probe used for Southern blots consisted of a pool of PCR products obtained from BACs covering the cluster by using degenerate primers corresponding to conserved regions of *V1ra/b* genes, as described[6]. *In situ* hybridizations were done with digoxigenin-labelled probes as described previously[22].

Phylogenetic tree

Was constructed as in ref. 7, except that V1rb11 was removed from the tree, because this sequence is not longer present in the updated Celera database.

Mice

Mutant and wild-type mice were in a 129/SvEv inbred background. They were housed in micro-isolator cages and given food and water *ad libitum*. Males and females were kept in separate cubicles after weaning. Behavioural experiments were performed during the dark phase of a 12:12 h light/dark cycle starting 2 h after the lights went off. Behavioural test sessions were recorded on videotape and subsequently analysed offline by an author who was unaware of the genotype of the mice. Males were weaned at 21 d old, and housed individually until behavioural testing, which was at 4–7 months. Females used for maternal aggression experiments were weaned at 21 d and housed in groups of five until they were mated, which was at 3–4 months.

Olfactory function

Mice were food-deprived for 18 h, after which an Oreo cookie was buried in the home cage of the subject under ~1 cm of bedding. The location of the cookie systematically varied across days.

Maternal aggression

Females were housed with 129/SvEv wild-type males for 15 d, after which the male was removed and females were singly housed until the day of the test. At parturition, pups were culled to 5 per litter. A single test for aggression was carried out 6–8 d after delivery. Each female was exposed for 10 min to an unfamiliar, group-housed, Swiss Webster mouse that had undergone olfactory bulbectomy (SWOBX). We recorded: (1) latency to the first attack; (2) total time attacking: cumulative time spent in any form of aggression (very rapid attacks that lasted less than 1 s were counted as 0.5 s); (3) number of attacks: number of times that the female bit the male; (4) number of fights: aggressive attacks involving intense body contact, with more than 3 consecutive seconds of tumbling, biting and wrestling; and (5) number of tail rattles.

Pup retrieval

Pups, 3 d old, were removed from their lactating mother for 5 min and returned to the home cage in a randomly distributed arrangement.

Ultrasound vocalizations

These were monitored with a bat detector (Petterson D200). A 3-min test began by placing a C57BL/6J wild-type female into the home cage of the subject.

Intermale behaviour

Aggressive behaviours were tested three times in a resident-intruder paradigm[18]. Each male was tested in his home cage against a group-housed SWOBX male intruder mouse for 15 min. The latency to the first attack, the number of attacks, the cumulative duration of aggression, the number of tail rattles, and the sexual behaviour by the resident mice towards intruder mice was scored. An attack was scored each time the resident male bit the intruder male. Whenever sustained attacks occurred with repeated biting, a new attack was not scored until the previous one has ceased for at least 2 s. The cumulative duration of aggression was scored as the total time that the resident mice spent in any of the following attacks: biting, chasing, wrestling, boxing and tail rattling. For sexual behaviour, the number of times the resident mounted the intruder was scored. The same experiments were repeated on a smaller scale using a castrated male as an intruder that had been painted with urine from gonadally intact males, obtaining similar results.

Male sexual behaviour

Male mice were tested 5 times (with an interval of 1 week between each test) for 30 min with a Swiss Webster female mouse in the male's home cage. Ejaculation was measured in the last behavioural session, over an extended period of 90 min. Females were ovariectomized and subcutaneously injected with 1 μg of oestradiol benzoate (48 h before the tests) and 500 μg progesterone (4–7 h before the tests) to ensure high sexual receptivity. The number of attempted mounts, the latency to mount, and the number of mounts with intromissions and ejaculations were recorded. Testosterone levels were measured with standard radioimmunoassay techniques.

Electrophysiology

Mice were used at 4–8 weeks old. Local field potentials (EVG) were recorded from the luminal surface of intact VNO sensory epithelia[9]. 2,5-dimethylpyrazine, 2-heptanone, *n*-pentyl acetate and isobutylamine were purchased from Aldrich; 2-*sec*-butyl-4,5-dihydrothiazole from Alfa Aesar; and farnesene from Bedoukian Research.

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The *ELF4* gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*

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Many plants use day length as an environmental cue to ensure proper timing of the switch from vegetative to reproductive growth. Day-length sensing involves an interaction between the relative length of day and night, and endogenous rhythms that are controlled by the plant circadian clock[1]. Thus, plants with defects in circadian regulation cannot properly regulate the timing of the floral transition[2]. Here we describe the gene *EARLY FLOWERING 4* (*ELF4*), which is involved in photoperiod perception and circadian regulation. *ELF4* promotes clock accuracy and is required for sustained rhythms in the absence of daily light/dark cycles. *elf4* mutants show attenuated expression of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), a gene that is thought to function as a central oscillator component[3,4]. In addition, *elf4* plants transiently show output rhythms with highly variable period lengths before becoming arrhythmic. Mutations in *elf4* result in early flowering in non-inductive photoperiods, which is probably caused by elevated amounts of *CONSTANS* (*CO*), a gene that promotes floral induction[5].

In *Arabidopsis*, a facultative long-day plant, the floral transition occurs earlier when plants are grown in long days (LD) than when they are grown in short days (SD)[6]. *elf4* mutants flower early in SD and are therefore impaired in their ability to sense day length (Table 1). In addition, *elf4* mutants have elongated hypocotyls and petioles (Table 1), particularly in SD. Both of these phenotypes may result from defects in circadian regulation[7,8]. A hallmark of circadian regulation is the persistence of robust, accurate rhythms for many days under conditions of continuous light (LL) or continuous darkness (DD). Circadian outputs, including rhythmic leaf movements and the expression of chlorophyll *a/b*-binding protein (*CAB*) and cold- and circadian-regulated (*CCR*) genes, are markers of clock function.

We introduced the luciferase reporter gene fusions *CAB–LUC* and *CCR2–LUC* into wild-type and *elf4* mutant plants. *CAB* rhythms in wild-type persisted in LL, whereas *elf4* mutants lost rhythmicity after one 24-h cycle (Fig. 1a). The arrhythmia of the

Table 1 **Flowering time and hypocotyl lengths of *elf4* mutants**

	Total leaf number*		Hypocotyl length (mm)†		
	LD	SD	CL	LD	SD
Wild type	7.8 ± 0.4 (30)	30.6 ± 5.1 (28)	2.9 ± 0.7	2.2 ± 0.7	4.7 ± 1.3
elf4	6.4 ± 0.9 (36)	10.1 ± 1.1 (23)	2.4 ± 0.4	3.1 ± 0.7	9.0 ± 1.9
elf3-4	4.9 ± 0.3 (36)	5.9 ± 0.3 (23)	2.8 ± 0.6	6.0 ± 0.2	10.2 ± 2.2

*Mean ± s.d. total leaf number was counted as rosette leaves plus cauline leaves. The number of plants is given in parentheses.
†About 15 plants were analysed in each trial.
CL, continuous light; LD, 16 h light/8 h dark; SD, 8 h light/16 h dark.