MOLECULAR DETECTION OF PHEROMONE SIGNALS IN MAMMALS: FROM GENES TO BEHAVIOUR

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The instinctive and species-specific behavioural response of animals to pheromones has intrigued biologists for a long time. Recent molecular and electrophysiological approaches have provided new insights into the mechanisms of pheromone detection in rodents and into the sensory coding of pheromone signals that lead to gender discrimination and aggressive behaviour.

SENSORY SYSTEMS

CONSPECIFICS
Individuals of the same species.

Animals have evolved specific communication strategies to identify and attract mates, and to discern the social status of CONSPECIFICS. In many species, this exchange of information involves the emission and detection of pheromones — a distinct and still poorly identified class of species- and gender-specific chemical cues that provide information about social and sexual status. The neuronal processing of pheromone signals within distinctive brain structures leads to marked changes in animal behaviour and endocrine status. The highly reproducible and species-specific character of the response to pheromones offers a unique opportunity to uncover the neural basis of genetically pre-programmed behaviours. Basic mechanisms of pheromone detection have been identified in various animal species, and here we will review recent investigations into molecular and neuronal sensory processing in the mouse. These studies have revealed a sensory strategy that is strikingly different from that of other chemosensory modalities, such as taste and olfaction.

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Evolution of pheromone signals

Pheromones are used by most species, from single-cell organisms to mammals. One type of cell–cell communication, known as 'quorum sensing', occurs when bacteria reach high population densities. They release auto-inducers that regulate bioluminescence, sporulation, mating, virulence factor expression and possibly biofilm formation[1]. Similarly, in yeast, peptide pheromones and molecular components of a G-protein signalling cascade that is required for the regulation of mating have been identified[2], illustrating the ubiquity of G-protein signalling pathways in chemosensory signal transduction, from single-cell organisms to complex mammalian sensory systems[3].

Analysis of pheromone-elicited behaviours in insects, including territory marking, colony identification, social hierarchy, reproductive status and mating rituals, has provided a conceptual framework for our understanding of pheromone communication. In insects, pheromone signals are detected by ultrasensitive receptors, and a given signal can elicit distinct responses according to the gender and social status of the recipient. Insect sex attractants comprise a well-defined group of pheromones that are detected in minute concentrations in the environment. For example, glypure and bombykol, the sex pheromones of the gypsy and silkworm moths, respectively, elicit responses in the male antenna at concentrations of only a few hundred molecules per square centimetre[4]. It has been estimated that the amount of compound that is present in one female moth could theoretically attract a billion male moths[4].

The study of chemical communication within insect societies has revealed the complexity of pheromone-evoked behavioural responses. In ants, the pheromone response shows an extreme specificity, which is a function of both the nature of the chemical cues — often released as blends — and the identity of the recipient[5].

Box 1 | Do humans have a functional vomeronasal system?

Is human behaviour or reproductive physiology affected by pheromones, and if so, are those responses mediated by a vomeronasal system? Despite extensive debate, these issues are still largely unresolved (reviewed in REF. 79). Behavioural responses to pheromonal compounds have been reported; for example, it has been suggested that pheromones are involved in synchronizing the menstrual cycles of women who live together[80,81], and compounds that purportedly elicit mood changes have been identified[82,83].

The existence of an embryonic structure that resembles a vomeronasal organ (VNO) and contains bipolar neurons is widely accepted[84], and a vomeronasal pit has been identified in some adults[85–87]. This pit contains few cells that resemble sensory neurons, although several studies have identified columnar cells with short microvilli that might function as chemoreceptors[86,88–91]. However, these cells have not been shown to possess axons that connect to the brain. A few bipolar cells within the pit stain positive for neuron-specific enolase (NSE) and the pan-neuronal marker PGP9.5 (REF. 86), but none stain positive for the olfactory marker protein (OMP, a marker of olfactory chemoreceptors) or S-100 (a marker expressed by glial cells that surround the vomeronasal nerve bundles in other organisms)[86].

Do humans possess an accessory olfactory bulb (AOB)? In a comparative description of the mammalian accessory olfactory system[92], it was reported that most adult non-aquatic mammals — including New World monkeys — possess an AOB, although it is absent in higher primates such as the Old World monkeys, apes, and humans. In humans and apes, the presence of a well-developed fetal AOB that regresses in later stages of development has been reported (reviewed in REF. 92) — a phenomenon that is strikingly similar to VNO regression in human fetuses.

Although evidence from anatomical analysis implies that the vomeronasal system is not functional in higher primates, recent evidence indicates that compounds thought to be pheromones might activate human vomeronasal receptor neurons and elicit stereotyped physiological responses[83,93–96]. These findings will need to be independently verified before the debate over the functionality of the human vomeronasal system can be resolved.

Recent advances in the molecular biology of pheromone detection in mammals might put the final nail in the coffin of this debate. Two large families of G-protein-coupled receptors that are expressed in the VNO and hypothesized to be pheromone receptors have been identified in mouse and rat. However, most of the human orthologues of those genes seem to be non-functional PSEUDOGENES[13,43,47]. In addition, mining of the human genome identified approximately 200 sequences with homology to the V1R family of vomeronasal receptors, and all but five are pseudogenes[97]. Of these five sequences (V1RL1–5), one (V1RL1) has been detected in the olfactory mucosa, although the nature of the expressing cells has not been investigated[98]. It is not yet known if V1RL1 is expressed in the human vomeronasal pit.

Recently, it was reported that the gene that codes for the ion channel TRP2 is essential for VNO function in mice[68,69], but is a pseudogene in humans[66]. Evolutionary analysis of TRP2 mutations in several primate species revealed that there is an absence of selective pressure on TRP2 function in Old World monkeys and apes[12]. This supports the hypothesis that the VNO was functional in the common ancestor of New World monkeys and Old World monkeys and apes, but became vestigial in the common ancestor of Old World monkeys and apes. The authors of this study propose the attractive hypothesis that pheromone signalling might have been replaced by other sensory modalities — in particular colour vision, which emerged at a similar evolutionary time.

The use of blends of chemicals might increase the specificity of recognition, or allow the transmission of more complex messages. Alarm pheromones, for example, are mostly composed of two or more chemicals that are used simultaneously to alert, attract and evoke aggression, and to induce different behaviours, as illustrated by slave-maker ant societies. Worker ants invade nearby nests, kill defending workers, and capture the pupae of the invaded nest. These captured pupae recognize the invading colony as their own, and develop into 'slaves' that maintain the nest of the invaders. Precise blends of ester acetates produced in the DUFOUR'S GLAND orchestrate these behaviours[6]. They are recognized as 'trail' or 'recruitment' pheromones by the slave-makers, marking the path to the target nest and eliciting attack behaviours, and they act as an 'alarm' signal in the resident nest, causing residents to panic and flee from the source of the signal. Moreover, they serve as a long lasting 'propaganda' signal that remains at the site of the invaded nest, preventing members of the invaded colony from returning.

Another important concept that has emerged from studies in insects is the distinction between two classes of pheromone signal — those that induce immediate or 'releaser' effects (for example, aggression or mating behaviours) and those that elicit long-lasting or 'primer' effects, such as physiological and hormonal changes[4]. In insects, these two types of response are mostly elicited by distinct sets of chemical cues (the priming and releasing pheromones), but in higher organisms, most pheromone responses seem to be composed of both short-and long-term components.

For vertebrates, aquatic and terrestrial environments each impose unique constraints on chemical communication. The aquatic environment is not particularly well suited for visual or auditory communication, but water is an ideal medium to disseminate aqueous chemosensory cues over large areas. Accordingly, many aquatic vertebrates use chemical signals for a wide range of purposes — to attract mates to distant nesting sites during spawning, to signal reproductive readiness and to regulate predator/prey interactions. Steroid hormone products have been identified as pre-ovulatory sex pheromones in goldfish, and they are released in various combinations, depending on the reproductive status of the releaser. These different pheromone blends can elicit varying degrees of male courtship and/or aggressive behaviour in the recipient[7]. Similarly, F-prostaglandins, which are important components of the post-ovulatory signal, elicit behaviours that are associated with male courtship[8].

Several species in a given environment might use structurally similar compounds as pheromones, so how is species specificity achieved? It is hypothesized that pheromone signals are usually not single compounds: instead, species specificity is conferred by variations in the ratios of single compounds that are contained in a blend of chemosignals[9]. The use of mixtures of compounds as pheromones is well documented in insects, and it fits well with the differing effects that are elicited by the multi-component pre- and post-ovulatory pheromones in goldfish.

In terrestrial environments, chemosignals can be either volatile or non-volatile. Accordingly, terrestrial vertebrates have two functionally and anatomically distinct olfactory systems: the main olfactory system (MOS), which is receptive to volatile cues, and the vomeronasal system, which is thought to process mostly non-volatile pheromones. This anatomical difference has been invoked to support the long-standing hypothesis that the vomeronasal system evolved as an adaptation to terrestrial life[10]. However, two independent lines of evidence contradict this hypothesis. First, as modern amphibians and amniotes both have vomeronasal organs (VNOs), it is likely that the last common ancestor

DUFOUR'S GLAND
A pheromone-secreting gland that is situated in the base of the sting apparatus in the worker ant.

PSEUDOGENE
A DNA sequence that is related to a functional gene but cannot be transcribed owing to mutational changes or the lack of regulatory sequences.

of modern amphibians and amniotes also had a VNO. Second, two families of fully aquatic, non-metamorphosing salamanders were shown to have vomeronasal systems, implying that the emergence of the vomeronasal system preceeded that of terrestrial life[11]. Although these findings have been used to discount the hypothesis that the vomeronasal system arose as an adaptation to terrestrial life, the two hypotheses are not necessarily mutually exclusive. They might indicate that the evolution of a vomeronasal system in aquatic species provided a selective advantage for terrestrial life, and consequently it was retained in many species of terrestrial vertebrates. Nevertheless, anatomical studies, and most recently molecular studies[12,13], indicate that the selective pressure to retain vomeronasal chemosensory input has been lost in higher primates, and that Old World monkeys, apes and humans might not have retained a functional vomeronasal system (BOX 1).

The mammalian vomeronasal system

Mammalian pheromones have been shown to elicit both long-lasting effects that alter the endocrine state of the recipient animal, and short-term effects on its behaviour. For example, detection of male pheromones by female mice results in an advance in the onset of puberty, induction of oestrus and termination of pregnancy, whereas detection of female pheromones causes a delay in the onset of puberty and suppression of oestrus. Modification of the hypothalamic–pituitary axis regulates the release of luteinizing hormone (LH) and prolactin (PRL), two hormones that are important for regulating these effects[14]. In general, physiological responses to male pheromones seem to be linked to an increase in LH and a reduction in PRL, whereas physiological responses to female pheromones are associated with an increase in PRL[15]. So, the vomeronasal system provides a neural pathway that links the periphery to the hypothalamus, and modulation of LH and PRL release through this pathway seems to provide the endocrine basis for the 'primer' effect of mammalian pheromones.

Mammalian pheromones also elicit rapid behavioural responses. These include the regulation of inter-male aggression, aggressive responses in lactating females, initiation of male ultrasonic vocalizations and copulatory behaviour, reinstatement of LORDOSIS in females, and parent–infant interactions. These immediate behavioural responses are also modulated by the endocrine status of the animal, which is itself under pheromonal control. For example, LH has a key role in regulating pheromone-mediated sex behaviours. LH-releasing hormone can reverse the effects of surgical removal of the VNO on copulatory attempts in males and lumbosacral stimulation-elicited lordosis in females[16,17], highlighting the crucial role of this hormone in regulating mating behaviour in both sexes.

Anatomy of the mammalian vomeronasal system. First described as a secretory organ of the mammalian nose by the anatomist L. Jacobson in 1813, the VNO is a bilaterally symmetrical, cylindrical organ encased in a bony capsule on the anterior nasal septum. The VNO is blind posteriorly, and depending on the species, opens anteriorly into either the nasal cavity or the oral cavity to allow entry of non-volatile chemical cues after direct physical contact of the snout with pheromone sources. Central connections of the VNO differ from those of the main olfactory epithelium (MOE) (FIG. 1). VNO neurons send a single, unbranched axon through an opening in the CRIBRIFORM PLATE of the skull to the accessory olfactory bulb (AOB), which lies in the posterior dorsal region of the main olfactory bulb (MOB), to which MOE neurons project. In turn, projection neurons of the AOB, known as mitral cells, project to discrete loci within the so-called vomeronasal amygdala, which includes the bed nuclei of the accessory olfactory tract and stria terminalis, and the posteromedial cortical and medial nuclei of the amygdala (reviewed in REF. 14). This set of projections is anatomically distinct from the multiple brain nuclei to which the MOB projects, and that form the primary olfactory cortex (FIG. 1). However, a subset of the tertiary projections from the MOS projects to the posteromedial cortical and medial nuclei, implying that integration of olfactory information from the MOS and vomeronasal system might occur in the amygdala[18,19].

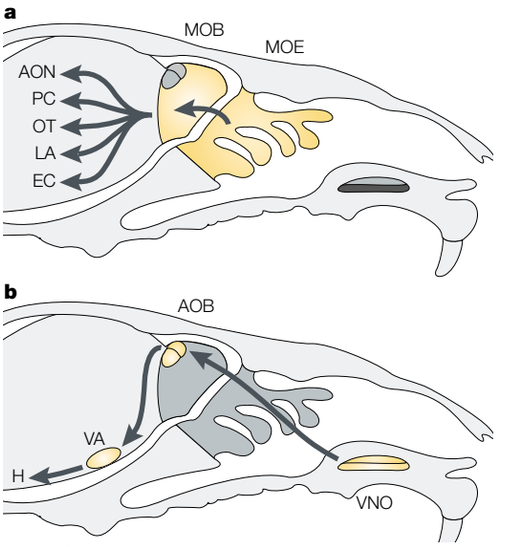

Figure 1 | **Functional and anatomical segregation of the two mammalian olfactory systems. a** | Olfactory sensory neurons in the main olfactory epithelium (MOE) are specialized for detecting small volatile odorants. The olfactory information is transmitted from the MOE to the main olfactory bulb (MOB), then to distinct brain nuclei that form the primary olfactory cortex and that include the anterior olfactory nucleus (AON), the piriform cortex (PC), the olfactory tubercle (OT), the entorhinal cortex (EC) and the lateral part of the cortical amygdala (LA). **b** | Information provided by pheromone signals is primarily processed by a distinct neural circuit. Pheromones are mostly detected by sensory neurons in the vomeronasal organ (VNO), a bilateral tubular structure in the anterior region of the nasal cavity. VNO axons project to the accessory olfactory bulb (AOB), which in turn transmits sensory information to the vomeronasal amygdala (VA) and then to specific nuclei of the hypothalamus (H), which are involved in regulating genetically pre-programmed physiological and reproductive responses.

LORDOSIS
A posture that is adopted by a female animal in anticipation of sexual intercourse, characterized by arching of the back to facilitate copulation.

CRIBRIFORM PLATE
One of the four components of the ethmoid bone in the base of the cranium. Its name derives from the Latin word for 'sieve', in reference to its spongy appearance.

Table 1 | **Small organic compounds with pheromonal activity**

Compound	Source	Effects	Synthetic compound active in water, CMU or both?	Binds to MUPs?	Other organisms in which pheromone previously identified	Refs
2-sec-butyl-4,5-dihydrothiazole (SBT) (1)	Male mouse urine. Production is testosterone dependent	Oestrous induction, intermale aggression and female attraction	CMU. Activity requires both SBT and DHB	Yes	Similar to male territory-marking compound used by grey and red duiker and two species of African antelope	26,33, 99–101, 106
2,3-dehydro-exo-brevicomin (DHB) (2)	Male mouse urine. Production is testosterone dependent	Oestrous induction, intermale aggression and female attraction	CMU. Activity requires both SBT and DHB	Yes	Male attractant of western pine beetle	26,33, 99–101, 106
α and β farnesenes (3)	Male mouse preputial gland. Production is testosterone dependent	Oestrous induction, intermale aggression	Both CMU and water	Yes	Trail marker of red fire ants, alarm phermones of aphids, defense substance of wild potato plants against aphids	26,27, 102
6-hydroxy-6-methyl-3-heptanone (4)	Male mouse urine	Puberty acceleration	Water	Yes		26
2-heptanone (5) *trans*-5-hepten-2-one (6) *trans*-4-hepten-2-one (**7**) *n*-pentyl acetate (8) *cis*-2-penten-1-yl-acetate (9) 2,5-dimethylpyrazine (10)	Female mouse urine. Production is dependent on adrenal gland function	Puberty delay	Active in both water and adrenelectomized female urine, in specific ratios with the other compounds isolated in this study			103
Dodecyl propionate (11)	Rat pup preputial gland	Maternal anogenital licking	Compound diluted in dichloromethane			104
(*Z*)-7-dodecen-1-yl acetate (12)	Female elephant pre-ovulatory urine	Male elephant sexual behaviour	Active in water		Turnip and cabbage looper, at least 100 species of lepidoptera	105

Numbers in brackets refer to chemical structures in FIG. 2. CMU, castrated male urine.

How does activation of the vomeronasal system lead to the behavioural outputs that are associated with pheromones? The tertiary projections of the VNO are primarily to hypothalamic areas that are involved in the regulation of reproductive and aggressive behaviours, such as the preoptic area, the ventromedial hypothalamic nucleus and the ventral premammillary nucleus (reviewed in REF. 14). These areas are often referred to as the 'neuroendocrine hypothalamus', and are known to control the release of hormones by the pituitary gland, thereby altering the endocrine state of the animal.

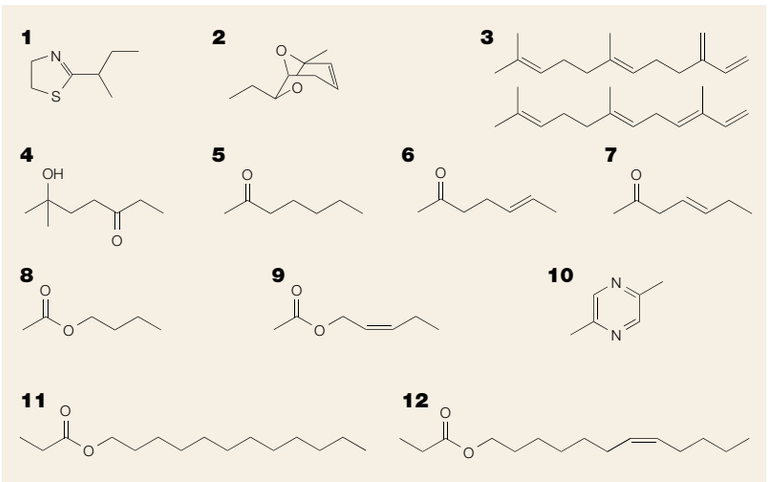

Figure 2 | **Structures of pheromones listed in table 1.**

The nature of mammalian pheromones

Although the identity of the mammalian pheromones is poorly understood, some compounds with pheromonal activity have been purified from complex mixtures, such as urine and anogenital gland secretions. Characterization of those compounds indicates that both proteins and small molecules might function as mammalian pheromones, and that production of those chemosignals is often hormonally regulated.

Early attempts to purify mammalian pheromones seemed to indcate that the proteinaceous components of complex secretions, such as urine and vaginal secretions, might carry chemosensory information. For example, the component in mouse male urine that regulates puberty acceleration in females was shown to be 'androgen-dependent, heat labile, non-dialyzable, precipitatable with ammonium sulphate and not extractable'[20]. Indeed, urine contains high levels of proteinaceous compounds known as MUPs (major urinary proteins), which are thought to act as olfactory binding proteins that 'deliver' small volatile molecules directly to the chemosensory receptor neurons. Similarly, analysis of hamster vaginal secretion, a potent male attractant, led to the identification of the protein aphrodisin, a molecule that elicits copulatory behaviours in male hamsters[21]. The pheromone effect was destroyed by proteolysis, but not by treatments that separated small molecule ligands from their protein carriers, implying at first that aphrodisin was itself an effective pheromone, although the activity of the recombinant protein has led to a reconsideration of this idea. Aphrodisin and MUPs

are members of the lipocalin family of proteins (reviewed in REF. 22). The lipocalins are a large family of structurally homologous proteins[23], with a molecular mass of between 15 and 30 kDa. Interestingly, the interior pockets that are formed by the tertiary structure of the MUPs form a highly apolar 'basket', which is ideally suited to bind and transport small, hydrophobic molecules that are thought to be pheromones[24], and small molecules that are present in urine have indeed been shown to bind to MUPs[25–27] (TABLE 1 and FIG. 2).

Separation of the MUPs from mouse urine by ion-exchange chromatography and mass spectrometry resolves up to 15 polymorphic MUPs, which are differentially expressed between lines of inbred mice[28,29]. Wild house-mouse populations show even greater heterogeneity in MUP expression[30,31]. There is evidence that the different MUP isoforms have different small molecule binding specificities[32], so individuals within a population could potentially be identified by the unique expression of a subset of polymorphic urinary proteins, each with a different small-molecule-binding specificity.

The issue of whether the MUPs, the small molecules bound to the MUPs, or the MUP–small molecule complexes are the active pheromones is still open to debate. Using advanced separation techniques, a handful of single molecules have been purified from urine and anogenital gland extracts and are implicated in various behavioural responses (TABLE 1). Remarkably, striking parallels between insect and mammalian pheromone communication have been revealed by the characterization of molecules that elicit behavioural responses in mammals, raising the possibility that some components of pheromone signalling systems have been conserved through evolution from insects to mammals (reviewed in REF. 33) (TABLE 1). Recently, it has been demonstrated that several of the purified single compounds activate VNO neurons at low concentrations, supporting the hypothesis that they are pheromonal components[34]. Moreover, various simple odorant compounds have been shown to activate the VNO[35,36], implying that the mammalian pheromones might act as pheromones or odorants by virtue of their processing by the vomeronasal or the olfactory system, respectively. In some species, the olfactory system has also been shown to carry subsets of pheromone responses.

Molecular biology of pheromone detection

What is the logic of pheromone detection in the mammalian VNO? How are pheromones detected by VNO neurons, and how is the specificity and the variety of the pheromone response generated? The characterization in the rat and the mouse of two large families of genes encoding putative pheromone receptors — the V1Rs and the V2Rs — and the identification of several essential components of the pheromone signalling apparatus have provided important insights into the molecular process of pheromone detection in rodents.

The dual repertoire of pheromone receptors. Early immunocytochemical studies on the AOB in various species revealed that the AOB is compartmentalized into an anterior and a posterior part[37–39]. Molecular heterogeneity was also demonstrated at the level of the vomeronasal epithelium. Viewed in cross section, the lumen is crescent shaped. A large blood vessel runs down the length of the VNO lateral to the lumen, and a concave neuroepithelium lines the medial side of the lumen (FIG. 3). Each bipolar receptor neuron extends a single apical dendrite through the supporting cells that line the lumen, and the receptor dendrites are bathed in fluid that is secreted by the vomeronasal glands at the dorsal and ventral tips of the lumen. Vomeronasal receptor neurons with cell bodies in the apical half of the sensory epithelium express the G-protein α-subunit $G\alpha_{i2}$ and project to the anterior part of the AOB, whereas neurons in the basal regions of the VNO neuroepithelium express the G-protein α-subunit $G_{o\alpha}$ and project to the posterior regions of the AOB[40–42]. Identification of the mammalian pheromone receptors further supported the idea of a functional dichotomy within the vomeronasal system.

A cloning strategy that was based on the differential screening of cDNA libraries prepared from single VNO neurons[43] led to the initial discovery of a multigene family that encodes putative G-protein-coupled receptors (GPCRs) — the V1Rs. Remarkably, the V1Rs do not show significant sequence homology with the olfactory receptors (ORs), but they share common motifs with the T2R family of bitter taste receptors[44]. The restricted expression of V1Rs in the VNO, such that each individual receptor gene is expressed by a small subset of neurons in the apical neuroepithelium, led to the hypothesis that the V1Rs represent a subset of the mammalian pheromone receptors. This idea has recently been confirmed by the observation that a mouse line lacking a cluster of V1Rs fails to respond to small molecules thought to be pheromones[45]. Furthermore, it has been shown that the response of vomeronasal neurons to 2-heptanone is mediated by the V1Rb2 vomeronasal receptor[46]. Another family of vomeronasal receptor genes, which are expressed in the $G\alpha_{i2}$ region and share distant similarity to both V1Rs and T2Rs, was later identified using a similar strategy. These receptors were named V3Rs[47]. Subsequent database mining of the completed mouse genome led to the identification of the entire repertoire of V1R genes, which comprises multiple divergent receptor families, including the original set of receptors that was identified in 1995 and the V3R family[48] (FIG. 3b). A total of 293 V1R sequences have been identified, although only 139 of them encode full-length OPEN READING FRAMES.

Phylogenetic analysis of ORs[49] and V1Rs[48] reveals the different evolutionary constraints that have been imposed on olfactory and pheromone detection, respectively. In contrast to the olfactory receptors, for which 228 closely related receptor families have been identified, the V1Rs comprise only 12 receptor families that are considerably divergent from one another, although the receptor sequences within each family show a high degree of similarity (FIG. 3). This molecular organization of the main olfactory system seems to be perfectly suited to the detection and discrimination of a vast number of stimuli in the

OPEN READING FRAMES
Stretches of DNA that are flanked by a 5′ initiation codon and a termination codon, which must be in the same reading frame. The presence of an open reading frame is one of the criteria that need to be fulfilled to show that a gene is functional.

AU: Units?

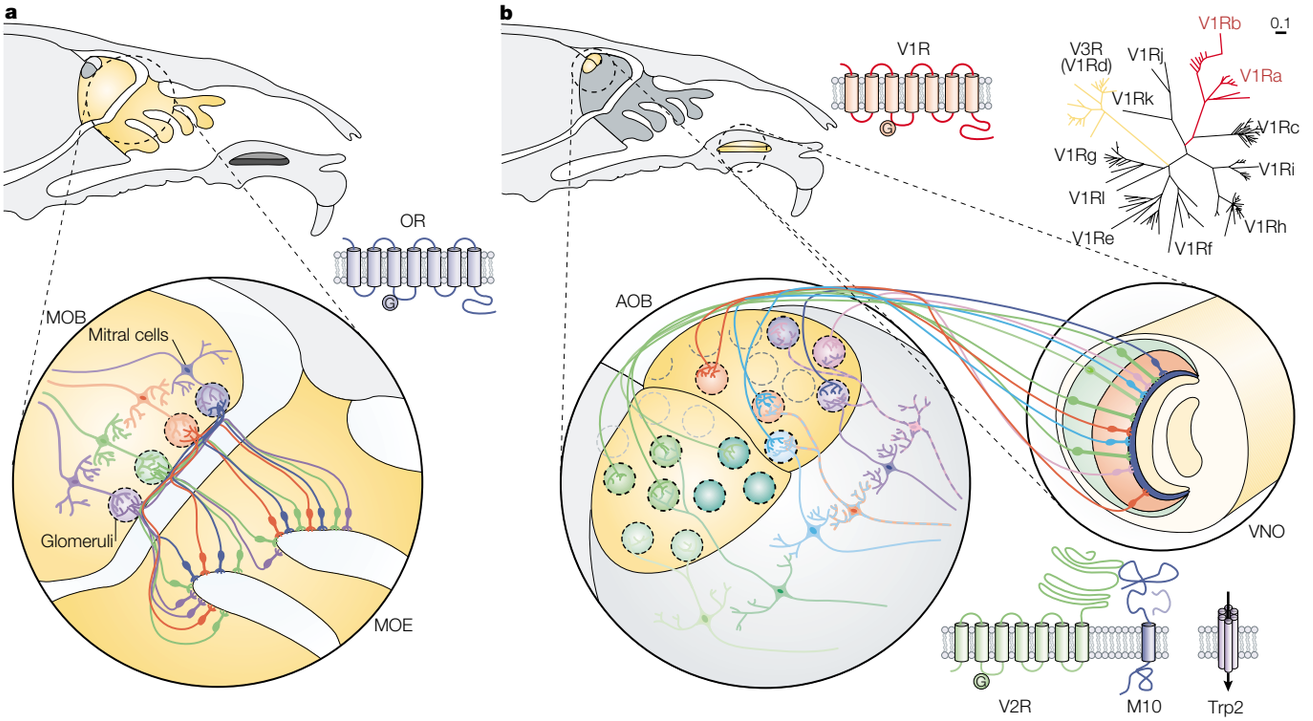

Figure 3 | **Cellular logic of sensory processing in the main and accessory olfactory systems. a** | In the main olfactory system, olfactory receptor neurons expressing a given olfactory receptor (OR) project to two bilaterally symmetrical, spatially conserved glomeruli in the main olfactory bulb (MOB), creating a topopographic map of olfactory receptor activation in the MOB. MOE, main olfactory epithelium. **b** | In the vomeronasal organ (VNO) two categories of sensory neurons can be distinguished anatomically and molecularly. VNO neurons in the apical half of the neuroepithelium (pink, red and turquoise) co-express the G-protein subunit $G\alpha_{i2}$ and a single receptor from the V1R class of pheromone receptors. The V1Rs are subdivided into 12 families, including the two families of receptors V1Rab and V3R (red and yellow in the upper right evolutionary tree). Vomeronasal receptor neurons in the basal vomeronasal epithelium (green) co-express the G-protein subunit $G_{o\alpha}$ and a single receptor from the structurally distinct V2R family of vomeronasal receptors. V2Rs interact with a non-classical major histocompatibility complex (MHC) class 1b molecule (M10) and the accessory molecule β2-microglobulin (β2m) to form a functional receptor complex. The activity of V1R- and V2R-expressing neurons requires expression of Trp2, a channel that is localized in the dendritic tips of the neurons (purple), and is a crucial component of the vomeronasal signalling cascade. Neurons expressing V1R and V2R receptors project to the anterior and posterior AOB, respectively, where they form multiple glomeruli in spatially conserved domains.

TRANSFERRIN
A metal-binding glycoprotein involved in ferric ion uptake into the cell. The pathway followed by transferrin bound to its receptor defines a classical recycling pathway.

CYCLOPHILIN
A protein that binds the immunosuppressant cyclosporin A.

CLATHRIN
A main structural component of coated vesicles that are implicated in protein transport. Clathrin heavy and light chains form a triskelion, the main building element of clathrin coats.

CALCITONIN
A 32 amino-acid polypeptide hormone that regulates calcium and phosphate levels in the blood.

environment, whereas the accessory olfactory system is specialized to detect a restricted subset of cues — the pheromones — that are emitted by conspecifics.

The V2Rs are another large class of putative pheromone receptors that are expressed in the basal $G_{o\alpha}$-positive zone of the VNO[50–52]. Unlike V1Rs, V2Rs have a large hydrophobic amino (N)-terminal extracellular domain, and share sequence similarity with the calcium sensing and metabotropic glutamate receptors. There are approximately 100 genes in the V2R gene family, and each V2R is expressed in a subpopulation of neurons within the $G_{o\alpha}$ zone of the neuroepithelium. In the rat, one V2R (G_o-VN2) was found to be expressed in a sexually dimorphic pattern — in the male, G_o-VN2 is expressed in the most apical portion of the $G_{o\alpha}$ zone, whereas in the female, it is expressed in a central portion of the $G_{o\alpha}$ zone[50].

The identification of two large families of V2R genes that are expressed in molecularly distinct and topographically segregated regions of the neuroepithelium strengthened the existing hypothesis that the rodent vomeronasal system consists of two segregated pathways, each of which detects a unique repertoire of pheromonal cues. This dichotomy raises the possibility that physiological responses result from selective activation of one or both of these pathways by the pheromones.

The multimolecular machinery of pheromone detection. A new and unexpected layer of complexity has recently been added to our understanding of the process of pheromone detection, with evidence for a functional association between the V2R pheromone receptors, the M10 and M1 families of non-classical major histocompatibility complex (MHC) molecules, and β2-microglobulin (β2m)[53]. The classical class Ia MHC molecules are expressed in association with β2m at the surface of most cells, and in the immune system their main role is to signal the presence of foreign invaders by presenting intracellular peptides to CD8-positive cytotoxic T lymphocytes[54].

Non-classical MHC class I molecules, which share sequence and structural homology with class Ia molecules but tend to show lower polymorphism and a more restricted expression profile, have been shown to participate in various immune and non-immune functions[55].

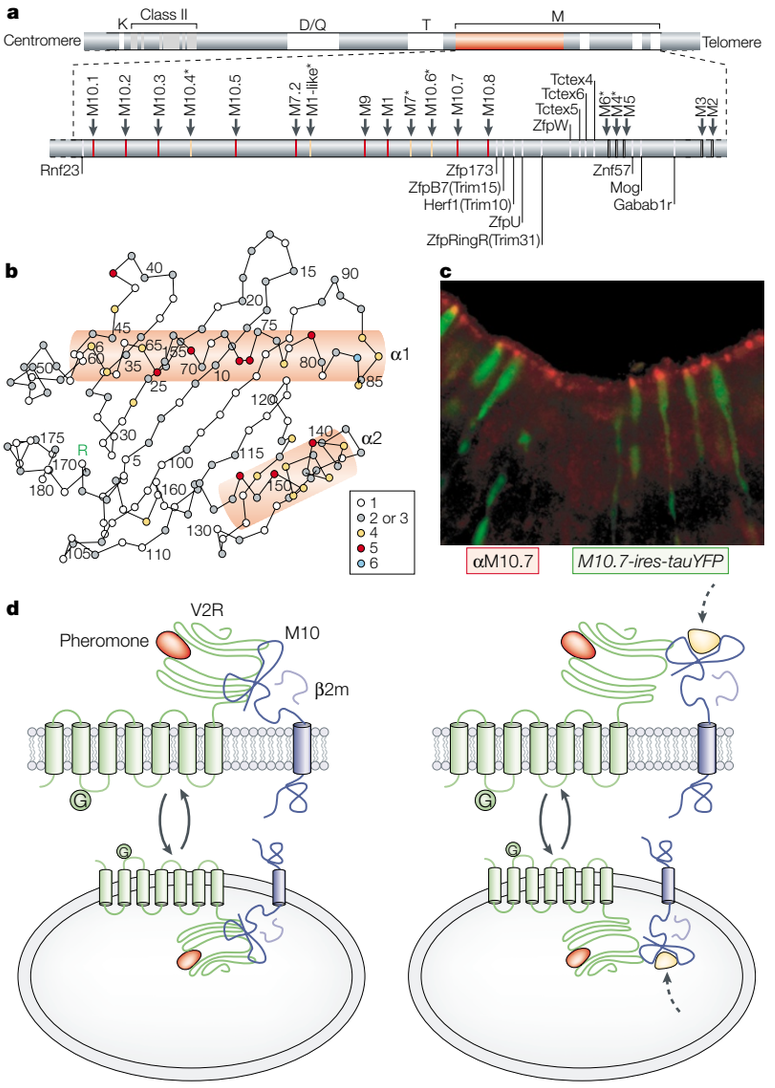

Figure 4 | **Genomic organization, expression and functional role of non-classical major histocompatibility complex (MHC) class I molecules in the vomeronasal organ (VNO).** **a** | The map of the H2M region on chromosome 17 localizes the genes that encode the M10 and M1 families of VNO-specific MHC class 1b molecules. Six out of eight M10 genes and three out of five M1 genes (red) probably generate functional transcripts. Pseudogenes are marked with an asterisk. Adjacent H2-M class I genes, and unrelated genes that are not expressed in the VNO, are shown in black, and pseudogenes are indicated with an asterisk. Adapted, with permission, from REF. 53 © (2003) Elsevier Sciences. **b** | Schematic representation of the variability of M10 proteins. The carbon backbone of an MHC class I molecule (HLA-A2) is colour-coded according to the number of different amino acids found at each position in the sequence of the six M10 proteins. The most variable residues are concentrated in the α1 helix and the beginning of the α2 helix. In the MHC class b molecule HFE, these areas, shaded here in orange, represent the site of interaction with the transferrin receptor, indicating a possible interaction of different M10s with distinct ligands or surface molecules. Figure courtesy of Kirsten Fischer-Lindahl. **c** | Immunostaining with the αM10.7 antibody (red) on fixed sections of adult VNO from an *M10.7-ires-tauYFP* transgenic mouse line (green) shows that M10.7 protein expression is confined to the dendritic tip of M10.7-expressing neurons. **d** | Two possible models for M10 interaction with the V2R pheromone receptor. The G-protein coupled V2R pheromone receptor interacts with its M10 molecular partner and β2-microglobulin (β2m) at the plasma membrane of VNO dendrite tips. Experiments *in vitro* and *in vivo* indicate that binding of M10 to V2R promotes the correct transport and targeting of the receptor to the membrane in the VNO (left panel). M10 might also control the recycling of pheromone receptor molecules to the surface (arrows). Sequence divergence among M10s indicates that they can recognize different ligands, the V2Rs (left panel) or additional small ligands (yellow in the right panel) originating from pheromonal sources or from intracellularly-derived molecules (dashed arrows).

Analysis of VNO gene expression led to the identification of two families of non-classical MHC class Ib molecules — M1 and M10 (FIG. 4 and REFS 53,56) — which are expressed exclusively in basal V2R-expressing neurons. The M1 and M10 gene families, which consist of 6 and 3 functional transcripts respectively, are clustered at the telomeric end of the mouse MHC (FIG. 4). Individual M10 and M1 genes are expressed in subpopulations of VNO neurons in the basal region of the mature neuroepithelium, such that individual neurons express only one or a few MHC class Ib genes.

Intriguingly, expression of a given M10 is strictly correlated with the expression of specific V2Rs, implying that the functions of the two types of molecule might also be correlated. Some class Ib molecules have been shown to present small ligands to immune cells in the groove made by the α1 and α2 domains, whereas others interact specifically with surface receptors — for example, the class Ib molecule hereditary haemochromatosis protein intereacts with the TRANSFERRIN receptor[57]. Sequence variability in the α1 and α2 domains of various M10s indicates that distinct members of the family might be able to recognize distinct ligands or receptor partners.

What is the functional role of M10 in the VNO? M10, V2R and β2m form a multimolecular complex that is localized to the dendritic tips of VNO neurons at the site of pheromone detection (FIG. 4), indicating that M10 might be involved either in receptor localization or more directly in the process of pheromone detection. *In vitro* experiments showed that M10s are required for proper expression of V2R at the cell surface, supporting a role for the complex in receptor trafficking. In further support of this hypothesis, immunohistochemical analysis of VNOs from *β2m*$^{-/-}$ mutant mice showed that localization of V2Rs to the dendritic tip of VNO neurons is severely compromised[53].

Accessory molecules that are required for chemosensory receptor transport have been identified in various organisms. The *Drosophila ninaA* gene product is a photoreceptor cell-specific CYCLOPHILIN that functions as a molecular chaperone that is required for folding the rhodopsin protein and escorting it through the secretory pathway[58,59]. In *Caenorhabditis elegans*, two gene products that are required for the proper localization of the odr-10 odorant receptor have been identified — the *unc-101* gene codes for an adaptor protein complex (Ap-1) μ1 CLATHRIN adaptor that is required for sorting and transport of odr-10 to the olfactory cilia[60], and *odr-4* codes for a membrane-associated protein that is required for proper folding, transport or localization of the odr-10 odorant receptor[61].

Interestingly, both the interaction of GPCRs with accessory molecules and the formation of GPCR heteromers often induce profound modifications in receptor specificity. This has been demonstrated for the T1R taste receptors[62,63] and the CALCITONIN receptor[64], and might also hold true for the V2R/M10 interaction. So, in addition to ensuring proper transport and stability of V2Rs at the VNO sensory terminals, the molecular association between M10 and V2R might significantly alter the

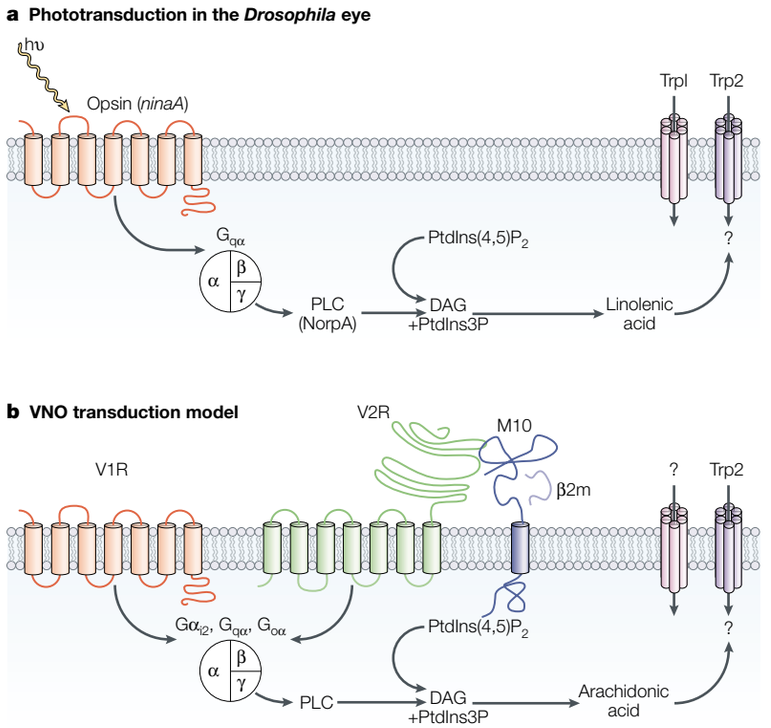

Figure 5 | **A model of sensory transduction in vomeronasal neurons. a** | In the *Drosophila* eye, photoisomerization of rhodopsin activates a Gα protein of the $G_{q\alpha}$ class, triggering a phosphatidylinositol-3-phosphate (PtdIns3P) signalling cascade that generates polyunsaturated fatty acids (PUFAs — linolenic acid in this case), which gate the cation-selective Trp and TrpI channels. DAG, diacylglycerol; PLC, phospholipase C; PtdIns(4,5)P_2, phosphatidylinositol-4,5-bisphosphate. **b** | In vomeronasal organ (VNO) neurons, a model of sensory transduction has been proposed that mirrors the signalling cascade in *Drosophila* photoreceptors. Binding of pheromone ligands to G-protein-coupled receptors (V1R and V2R) results in stimulation of a heterotrimeric G protein, which in turn triggers an PtdIns3P-signalling cascade that generates the PUFA second messengers (in this case, arachidonic acid) that gate the Trp2 cation channel. Recent electrophysiological characterization of hamster VNO neurons indicates that an unidentified channel (pink) might either be directly involved in pheromone transduction or amplification of the primary sensory response. β2m, β2-microglobulin; hυ, photons.

mechanism and specificity of pheromone recognition, adding an interesting new twist to the molecular mechanism of pheromone detection.

Transduction of pheromone signals

Neurons in the MOS and vomeronasal system use distinct signalling components to transduce chemical stimuli. The main components of the olfactory transduction cascade, G-protein olfactory subunit (G_{olf}), adenylyl cyclase type III (ACIII) and olfactory cyclic nucleotide-gated channel subunit 1 (OCNC1), are not expressed in vomeronasal sensory neurons[65]. Instead, Trp2, a cation channel belonging to the transient receptor potential (TRP) family of ion channels, is highly expressed in VNO neurons. This channel is specifically localized to the microvilli, the proposed site of pheromone transduction, implying a direct role in the VNO signalling cascade[66]. Trp2 might represent the primary conductance that is activated by pheromone signals, or could mediate a secondary amplification of the sensory response.

PATCH CLAMP
Technique whereby a small electrode tip is sealed onto a patch of cell membrane, making it possible to record the flow of current through individual ion channels or pores within the patch.

The identification of Trp2 led to a model of VNO signal transduction that parallels the *Drosophila* phototransduction cascade[66] (FIG. 5). According to this model, G-protein activation by vomeronasal GPCRs triggers a phospholipase C (PLC)-dependent cascade, which in turn directly activates either Trp2 or another associated conductance. Accordingly, the two families of GPCRs, V1R and V2R, are co-expressed in topographically segregated and non-overlapping populations with the G-protein α-subunits $G\alpha_{12}$ and $G_{o\alpha}$, respectively. Although a direct role in vomeronasal sensory transduction has not been shown for either of those G proteins, stimulus-induced electrical activity in vomeronasal neurons is completely abolished by treatment with a pharmacological agent that inhibits PLC activity, showing that PLC has a crucial role in vomeronasal sensory transduction[67]. Moreover, in agreement with the proposed model of VNO transduction, the genetic ablation of Trp2 in the mouse markedly impairs the pheromone-evoked response[68,69].

What is the mechanism of Trp2 activation? PLC activity results in the cleavage of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2), leading to an increase in the intracellular concentrations of the second messengers inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG), both of which have been implicated in TRP activation[70]. However, it has recently been reported that stimulus-induced calcium transients in VNO neurons are not affected by depletion of internal stores or by treatment with inhibitors of Ins(1,4,5)P_3 receptors[71]. Instead, stimulus-induced VNO responses seem to depend on PLC and DAG lipase, and calcium transients in VNOs were elicited by application of polyunsaturated fatty acids, including linoleic and arachidonic acid[71] (FIG. 5).

Interestingly, PATCH-CLAMP recordings of hamster VNO neurons identified the abundant expression of a Ca^{2+} activated non-selective cation channel with properties that are consistent with a direct role in VNO sensory transduction or with an indirect function in amplifying the primary sensory response[72]. The relationship and possible identity between this conductance and Trp2 remain to be investigated.

VNO signalling to the brain

How is information about sex, social dominance and individual identity represented by the signalling from the VNO to the brain? The unexpectedly large repertoire of pheromone receptors that are grouped into several divergent receptor families indicates that the pheromone-evoked response might involve complex patterns of activity across the neuronal population. Accordingly, several experimental approaches were designed to observe the activity of a large number of neurons in response to pheromonal stimuli.

Using a flat array of 61 extracellular electrodes[67], the changes in spike activity of large subsets of VNO neurons in response to natural stimuli was recorded in the mouse (FIG. 6). This study revealed several essential features of VNO neuronal activation and provided a direct tool to investigate the complex sensory recognition that is

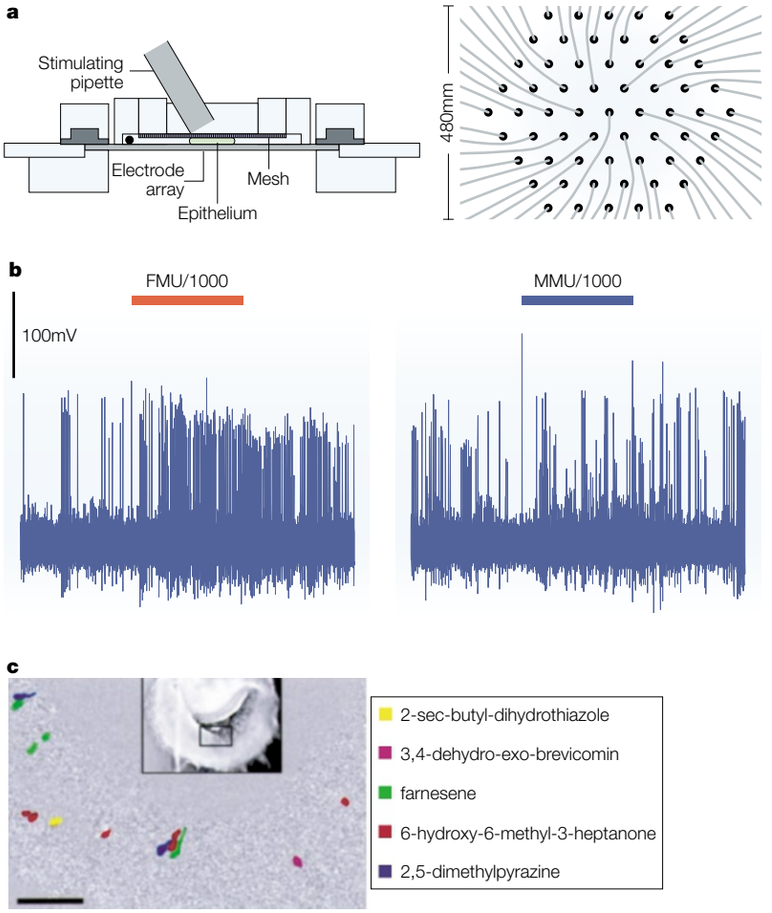

Figure 6 | **Responses of vomeronasal neurons to complex mixtures and single pheromones. a** | Multielectrode recording of vomeronasal organ (VNO) activity. VNO neuroepithelium, dissected from the blood vessel, connective tissue, and nerve layer, is held in place with wire mesh on a 61-electrode array (schematically represented in the upper right inset) with the microvilli facing up. The preparation is continuously superfused with fresh Ringer's solution, and stimuli are locally applied using a stimulating pipette. Figure courtesy of Tim Holy. **b** | The selective responses of an individual VNO neuron to diluted female urine (FMU) and diluted male urine (MMU) using the microelectrode array recording apparatus described in (**a**). The increase in the spiking response of this neuron shows selectivity for pheromonal compounds present in female urine, but not in male urine. Figure courtesy of Tim Holy. **c** | A map of vomeronasal neuron activation produced by successive application of individual phermones. Responses of vomeronasal neurons in a slice preparation were detected by confocal intracellular calcium imaging. Colour-coded responses of individual neurons to each of the compounds listed (presented at 10^{-6} M) shows that each pheromonal ligand activates a unique, non-overlapping subset of vomeronasal neurons. Reproduced, with permission, from *Nature* REF. 34 © (1999) Macmillan Magazines Ltd.

involved in the pheromone-evoked response, including the discrimination of species, sex, familial status and individual differences between animals. It seemed that as many as 40% of the recorded neurons responded to the complex pheromonal stimuli that are present in urine by increasing their spiking firing rate (FIG. 6). Moreover, subsets of VNO neurons were activated selectively by either male or female urine, whereas the response of others was independent of the sex of the donor animal[67], and might instead provide information about other characteristics of the donor. Finally, unlike olfactory neurons, which adapt to sustained stimulus applications by altering their sensitivity, little or no adaptation was seen in the VNO responses. This result indicates that the ability to retain sensitivity to variations in pheromone concentration over a wide range of background intensities might not be required.

The optical imaging of neuronal activity in VNO slices offered a distinct experimental strategy to observe the response of large populations of sensory neurons to pheromonal stimuli[34] (FIG. 6). This study showed that neurons in the apical, $G\alpha_{i2}$-positive region of the VNO respond to small molecules that were identified as pheromones. Remarkably, in contrast to the broad and concentration-dependent tuning of the ORs, vomeronasal receptor activation seems to be ultrasensitive and extremely specific. Concentrations as low as 10^{-11} M were sufficient to activate a distinct population of VNO neurons, and an increase in the stimulus concentration did not alter the neuronal tuning. Furthermore, specific compounds were shown to activate about 0.2–3% of the VNO neuronal population, a number approximately equal to the number of neurons that express a given V1R. This implies that, in contrast to ORs, V1Rs are narrowly tuned to minute concentrations of pheromonal compounds. These results are consistent with the idea that, in contrast to the MOS, which is designed to recognize the chemical world at large, the vomeronasal system has evolved to detect a small subset of chemosensory cues with extreme sensitivity and specificity.

Brain representation of pheromone signals

How is activation of the pheromone receptors represented in the brain? Genetic techniques similar to those used in the MOS revealed striking patterns of glomeruli in the AOB, such that neurons expressing a given vomeronasal receptor project to multiple glomeruli that occupy spatially conserved regions within the AOB[73–75] (FIG. 3). Although the function of these domains is not known, it is tempting to speculate that they constitute individual processing units, which are involved in integrating behaviour-specific chemosensory information. In contrast to the MOS, in which glomeruli are composed of fibres from neurons that express the same receptor (FIG. 3), it has been reported that glomeruli in the accessory bulb might receive inputs from several receptor types[73].

How is the map of pheromone receptor activation represented in higher brain centres? In the MOS, the secondary projection neurons (mitral cells) are organized such that each mitral cell innervates only one glomerulus, so the topographical map of odorant receptor activation in the MOB is largely retained and transmitted to higher brain centres. Similarly, it was recently reported that individual mitral cells might innervate multiple glomeruli that are formed by neurons expressing the same receptor[75]. These results indicate that the divergent pattern of glomerular position in the AOB might at least partially be rendered convergent at the level of the mitral cells, and that mitral cells might transmit information from a single receptor type to higher brain centres. However, this study does not preclude that there are more complex patterns of mitral cell dendritic projections.

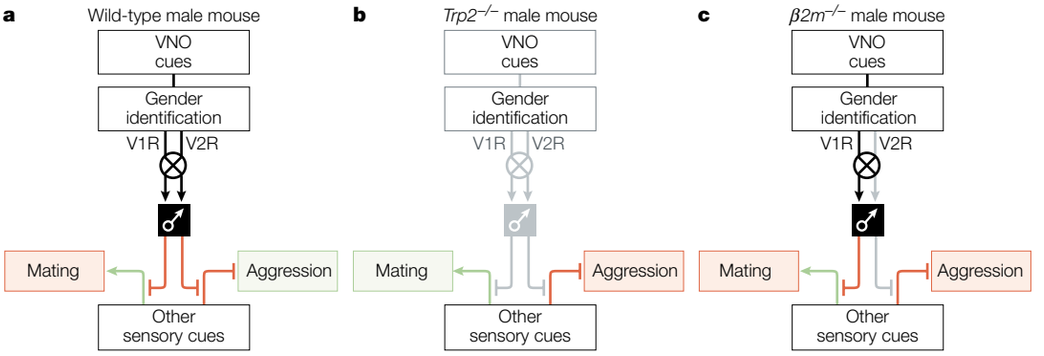

Figure 7 | **Role of the mouse vomeronasal organ (VNO) in gender discrimination. a** | In wild-type mice, display of gender-appropriate responses is ensured by VNO activity. The detection of male pheromones by a male results in inhibition of mating (red) and initiation of aggressive behaviours (green). **b** | Behavioural analysis of *Trp2*$^{-/-}$ males indicates that non-VNO-related sensory inputs, such as olfactory, auditory, tactile or visual cues, trigger mating behaviour (green) irrespective of the gender of the encountered mouse. So, in the absence of VNO activity, mating is the default behaviour of the male with a conspecific, and aggressive responses are suppressed (red). **c** | Behavioural analysis of *β2m*$^{-/-}$ male mice indicates that V2R signalling is involved in the regulation of aggressive responses, but does not affect gender-specific reproductive responses. In the absence of V2R activity, appropriate gender-specific reproductive behaviours remain intact — male–male mating is inhibited and aggressive responses are suppressed.

How is pheromonal information encoded in the mitral cells? Using an array of electrodes implanted in the mouse AOB, Luo and colleagues recently recorded mitral cell activity in freely moving mice[76]. This 'tour de force' opened the window into real time decoding of pheromonal stimuli by the brain in the behaving animal, and it showed that mitral cell responses to complex pheromonal cues seem to be extremely specific to the strain and gender of the stimulus. Furthermore, both activation and inhibition of mitral cell activity was observed, such that each mitral cell displayed a unique repertoire of activation and inhibition by specific stimuli, whereas some mitral cells were first activated and then inhibited by the same stimulus. These results show a striking resemblance to recordings from the brains of insects in response to pheromones[77], and they indicate that the sensory cues that provide information about sex and species are integrated by the vomeronasal system, perhaps by local inhibitory circuits in the AOB.

From VNO activation to behavioural changes

How is VNO signalling converted into behavioural changes? How are the specificity and variety of the pheromone response generated? The surgical ablation of the VNO or the AOB in rodents has been shown to impair mating and territorial defence, implying a crucial role for pheromones in gender and social recognition (reviewed in REFS 14,78). However, clear differences in the degree of behavioural defects were observed in different species and, to some extent, in various reports of the same experiment. This can be easily explained by the behavioural variability between different species and strains, and by the inherent difficulty of performing this surgical procedure.

Genetic ablation of Trp2 in the mouse has provided a new experimental system to assess the requirement of Trp2 function in VNO signalling and to investigate directly the repertoire of VNO-mediated sensory responses and behaviours[68,69]. First, it seems that the Trp2 deficiency markedly impairs the sensory activation of VNO neurons by urine pheromones, thereby confirming the crucial role of Trp2 in the VNO signal transduction cascade. In addition, the absence of pheromone detection mediated by VNO signalling has striking behavioural consequences. *Trp2*$^{-/-}$ male mice seem to be unable to recognize the sexual identity of their conspecifics. For example, they fail to show the pheromone-evoked aggression towards male intruders that is normally seen in wild-type males and, remarkably, they display courtship and mounting behaviour indiscriminately towards both males and females. These data contradict the established idea that VNO activity is required for the initiation of male–female mating behaviour in the mouse, and instead they imply a crucial role in ensuring the establishment of — or a direct involvement in — sex discrimination (FIG. 7). Defects in maternal aggression, male territory marking and recognition of social dominance also seem to be impaired in the *Trp2*$^{-/-}$ mouse line[69].

The behavioural phenotype of the β*2m*$^{-/-}$ mouse line, in which only V2R-related detection is likely to be affected in the pheromone sensory response, has provided the first indication of distinctive roles for the apical and basal neuronal compartments of the VNO[53] (FIG. 7). Remarkably, the β*2m*$^{-/-}$ mouse line does not display male–male aggression, as previously described in the *Trp2*$^{-/-}$ mouse line[68,69]. However, in contrast to the behaviour of *Trp2*$^{-/-}$ males, in which the entire VNO sensory detection system is impaired, β*2m*$^{-/-}$ males — which are likely to retain fully functional V1R-related pheromone detection — do not show sexual attempts towards other males, indicating that sex discrimination is normal. So, the V1R- and V2R-positive VNO neuronal populations might have distinct roles in controlling sex discrimination and pheromone-induced aggression, respectively.

How do individual receptors of the V1R or V2R class participate in sex discrimination and aggression? A mouse mutant line, which was deficient for a small subset of V1Rs comprising 12% of the V1R repertoire

(16 V1Rs), was constructed to address the role of distinct receptors in mediating specific behavioural arrays[45]. The mutant line shows a modest but complex phenotype, with partial alteration of maternal aggression and male sexual behaviour. However, some of the defects are opposite to those observed in the *Trp2*$^{-/-}$ mutant line — for example, there is a decrease in male–male mounting attempts — indicating that the deletion of a small random fraction of the V1R repertoire generates a 'corruption' rather than a physiological impairment of the pheromone response.

Concluding remarks

The identification of key players of the pheromone detection apparatus, including the V1R and the V2R receptor families, the M10 and M1 receptor escorts and the Trp2 ion channel, has provided the molecular framework for our understanding of the pheromone-evoked response in the VNO. In addition, new imaging and recording technologies have offered a new opportunity to examine the pheromone-evoked response of large populations of neurons in the VNO, and more recently in the brain.

Molecular and electrophysiological approaches have both uncovered an unexpected diversity and complexity that underlies the function of the mammalian vomeronasal system. For example, considering the small number of behavioural and endocrine responses that have been observed, the repertoire of pheromone receptors is surprisingly large. The detection of natural stimuli leads to the activation of large populations of sensory neurons, yet in the VNO, each receptor type shows a narrow tuning for simple compounds present at minute concentrations, and mitral cells of the AOB seem to be similarly tuned to pheromonal stimuli from specific sources. Finally, genetic analysis of mutant mouse lines that are deficient in key VNO signalling components has provided important insights into the physiological role of the vomeronasal system — in particular, its exclusive role in gender identification.

Future studies will require us to associate the different elements of the current puzzle. What is the importance of the myriad chemicals detected by the VNO, how do they carry information about sex, species and individual identity of the animal, and how do they relate to the different receptor families and mitral cell activation? Furthermore, the pheromones seem to act not as simple releasers of mating or aggressive behaviours, but rather as essential regulators of inputs from other sensory organs, ensuring the sex specificity of the behavioural response. So, in addition to the intriguing nature of a brain circuit that is designed to recognize sex, one wonders how the vomeronasal system impinges on the function of the other sensory networks. The pheromone signalling of mammals still retains wonderful mysteries.

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