

Pheromones, binding proteins and receptor responses in rodents

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Abstract

Their well-developed chemical communication systems make rodents popular in research that aims to understand the connections between genes, hormones and behaviour. Structural identification of several pheromones in mice, rats and hamsters now makes it feasible to employ their synthetic analogues in probing olfactory neurons and in the study of various pheromone–protein interactions in intimate detail.

Introduction

Even the most casual observers of animal behaviour become aware of the widespread phenomenon of chemical signalling. Various insects seek their mating partners over a distance, snakes find their prey through olfaction, and even seabirds use their sense of smell in foraging. Most mammals are known to scent-mark their territories and to avoid the places marked by stressed or alarmed conspecifics. These are just a few examples from Nature of incidences when different individuals emit and receive chemosignals, some with incredible sensitivity of perception.

The chemical communication field, as a relatively new scientific endeavour, represents a true ‘renaissance science’ with many interdisciplinary connections. As genetics, hormonal differences, social imprinting, health status, environmental stress, dietary modification, etc., all seem to have a profound influence on how chemosignals are emitted and perceived in the overall context of competition for reproductive opportunities, many previously separate scientific disciplines now find a overlapping area. What used to be almost exclusive areas of psychology and behavioural science (preoccupied with the social interactions subsequent to perception of an uncharacterized chemosignal and ‘olfactory memory’) and physiology, with its main emphasis on hormonal regulations, can soon be supplemented by a substantial input from chemistry and biochemistry.

While ‘chemical connection’ of physiology and behaviour were clearly brought to chemists’ attention through the classical studies on insects (when the term pheromone was coined during the 1950s), considerably less attention has been paid to mammalian chemical signals. Some discussions of the following two decades even discouraged the use of this term in the context of mammalian reproductive behaviour. At present, various scientists use the term ‘pheromone’ in the context of mammals with considerably less

hesitation. This can be at least partially attributed to (i) the recent ground-breaking research that has identified the ubiquity of receptor proteins found in olfactory neurons, (ii) an improved understanding of the ‘chemically selective’ vomeronasal organ (VNO), and (iii) the demonstration that distinct behavioural and hormonal responses can be caused by a single chemical substance or a relatively simple synthetic mixture in mammals.

While the field of chemical ecology is replete with examples of diversity in the way mammalian chemosignalling occurs, the emerging molecular details of pheromone release and perception have been largely available through work in rodents, particularly house mice, rats and hamsters. The laboratory-bred mice are now sufficiently well defined genetically to meet various demands of biomedical research, so that it is not surprising to view them as the animals of choice in behavioural genetics. The recent availability of mutant and gene-knockout mice shows considerable promise for the step-by-step elucidation of the pathways leading to pheromone biosynthesis, as well as of signal transduction following the primary events at olfactory neurons. Moreover, the overwhelming reliance of mice on chemical communication [1] and the pioneering biological studies implicating the effects of olfaction on the endocrine function [2–6] all strongly favour the use of the house mouse in chemical, biochemical and neurobiological studies. The Norway rat and several hamster species have long been popular with psychobiologists for their learning capabilities and information processing by the brain. Importantly, rodents possess well-developed and functional VNOs, providing an easily accessible site for studying pheromone–receptor interactions [7,8].

Chemical nature of mammalian pheromones

Elucidating the structures of mammalian pheromones is usually more involved than the corresponding studies in insects. While the pheromone-rich glands that are relatively easily identified in insects usually contain abundant amounts


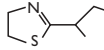
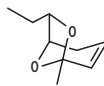
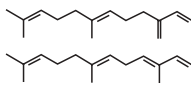
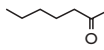
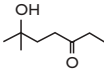
Key words: chemical signal, mammalian communication, olfaction, vomeronasal organ

Abbreviations used: MOE, main olfactory epithelium; MUP, major urinary protein; VNO, vomeronasal organ.

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Figure 1 | Structure, origin and function of prospective pheromones

Source: reference [7].

Name	Chemical structure	Origin	Possible chemosignalling function in female mice	Detection threshold
2,5-dimethylpyrazine		Female urine	Puberty delay	10^{-6} – 10^{-7} M
2-sec-butyl-4,5-dihydrothiazole		Male bladder urine	Oestrus synchronization Puberty acceleration	10^{-10} – 10^{-9} M
2,3-dehydro- <i>exo</i> -brevicomine		Male bladder urine	Oestrus synchronization Puberty acceleration	10^{-10} – 10^{-9} M
α - and β -farnesenes		Male preputial gland	Puberty acceleration	10^{-11} – 10^{-10} M
2-heptanone		Female or male urine	Oestrus extension	10^{-11} – 10^{-10} M
6-hydroxy-6-methyl-3-heptanone		Male bladder urine	Puberty acceleration	10^{-8} – 10^{-7} M

for chemical analysis, mammalian chemical signals are typically embedded in a very complex biological matrix (urine or a glandular secretion) containing dozens of seemingly 'non-pheromone' metabolites. The chemical analysis must first ensure separation of the chemosignals from the rest of the uninteresting metabolites. How do we know which of the many excreted substances have the pheromonal activity? In our laboratory, we often use the known effects of hormones on pheromone excretion to identify plausible pheromone candidates. Assuming that a pheromone is a volatile substance, capillary GC–MS is capable of developing differential metabolic profiles of volatiles for the animals under different endocrine status or behavioural manipulation. Gross quantitative differences in the excretion of volatiles alert us to the compounds that need structural elucidation and, subsequently, evaluation through biological assays. The above-mentioned utilization of the hormone–pheromone axis has often (but not always) led us to the eventual implication of singular substances in changing physiological responses and behaviour. In our early work, the sexually dimorphic profiles in the red fox [9] led to the observation of induced marking behaviour by synthetic mixtures of two sulphur-containing compounds in the natural habitat [10]. Although it could hardly be referred to as a 'pheromone', this mixture represents one of the first uses of synthetic chemosignals to induce a characteristic animal behaviour. A similar approach was later used in implicating structurally both male and female mouse signals responsible for aggressive behaviour [11], sexual attraction [12,13], dominance signalling [14], oestrus induction [15,16], puberty acceleration [17,18] and puberty delay [19]. In the more recent studies, aversive responses of male rats to androgen-dependent chemosignals (W. Ma,

M.V. Novotny and J.R. Alberts, unpublished work) and attraction of female hamsters to a flank gland major volatile constituent (W. Ma, R. Sanders, D. Wiesler, J. Song, and M.V. Novotny, unpublished work) were verified using the same general approach.

It is clear that a comprehensive approach to identification of mammalian chemosignals is a multidisciplinary task involving (i) bioanalytical methodologies of separation science and MS (on occasion, additional structural tools may become applicable), (ii) structural elucidation through spectral interpretation and verification of a candidate substance through a synthetic, authentic sample and (iii) the use of this synthetic compound (alongside the natural stimulus) in biological testing. Testing of the pheromone candidates in mammals, compared with the similar objectives in the insect studies, typically represents a more difficult task with the former, owing to a response 'plasticity' of mammals. Some behavioural and endocrinological bioassays can be tedious, necessitating extensive experimentation and careful statistical evaluation.

The pheromonally active compounds in the house mouse have largely been identified as volatile substances that tend to bind to the proteins excreted into urine. The bladder urine and preputial gland contents are currently known as the most significant sources of pheromone activity. As seen in Figure 1 [7], the mouse pheromones that have been identified thus far belong to several chemically distinct classes. The nitrogen-containing 2,5-dimethylpyrazine and 2-*sec*-butyl-4,5-dihydrothiazole are strongly odoriferous compounds, as are the sesquiterpenes, α -farnesene and β -farnesene. While 2-heptanone is a fairly common metabolic product, 6-hydroxy-6-methyl-3-heptanone and 2,3-dehydro-*exo*-brevicomine are

structurally unusual odorants. 2-Heptanone and 6-hydroxy-6-methyl-3-heptanone are carbonyl compounds, as are the recently identified ethyl-substituted ketones (W. Ma, M.V. Novotny and J.R. Alberts, unpublished work) found in the male rat, and an unsaturated ketone identified as the male sex attractant in golden hamsters (W. Ma, R. Sanders, D. Wiesler, J. Song and M.V. Novotny, unpublished work). All of them are structurally dissimilar to the hormonally dependent compound found in the urine of pine voles [20], California mice [21] and deer mice [22], indicating that species-to-species differences among different rodents are substantial.

The structural similarities between the putative mammalian chemosignals and the pheromones identified previously in the insect-world are quite striking. The farnesenes [23,24] and 2-heptanone (reviewed in [25]) have been implicated previously as insect pheromones. There is only one double bond distinguishing the male mouse pheromone dehydro-*exo*-brevicommin from a bark beetle pheromone [26], brevicomin. This is a peculiar case, as the biosynthetically uncommon cyclic ketal skeleton is apparently shared by evolutionarily very distant species; the house mouse shares this molecular arrangement with no other rodent. In addition, the elephant pheromone (*Z*)-7-dodecen-1-yl acetate is also known to be an insect pheromone [25].

Can the same chemical compounds impart a different message in a behaviourally or endocrinologically different context? Apparently so. The male-originated dehydro-*exo*-brevicommin and 2-*sec*-butyl-4,5-dihydrothiazole potentiate aggression in male mice [11], while being attractive odours to females [12] and oestrus-inducing pheromones [15,18]. The sesquiterpenic α -farnesene and β -farnesene signal dominance in males [14], but the same compounds presented in dilute water solution are also attractive to females [13]. Likewise, the adrenal-mediated metabolite 2,5-dimethylpyrazine profoundly effects sexual maturation of females [19] and has a disruptive effect on their reproductive fitness [27]; it may have a very different signalling function in males [28].

Can different chemical entities cause the same profound olfactory biological response? Oestrus induction/synchronization can be caused by the bladder-originating dehydro-*exo*-brevicommin and 2-*sec*-butyl-4,5-dihydrothiazole [15,18], and the farnesenes that are excreted through the preputial gland [16]. Puberty acceleration in juvenile females can be induced independently by several testosterone-dependent urinary volatiles [18]. We often find adaptive redundancies in Nature [29], i.e. the same biological phenomenon can be controlled by a defined, albeit different, set of chemical signals.

Pheromone-protein interactions

Mammalian pheromones released into their environment can readily reach their target tissue, either the main olfactory epithelium (MOE) or the VNO. Both target tissues are lined with an olfactory neuroepithelium that contains membrane-bound receptor proteins, which comprise the

largest known family of G-protein-coupled receptors in mammals. The number of mammalian olfactory receptors [30–32] that has been found has been astonishing, but not unreasonable. Apparently, they comprise the heart of the ‘machinery’ for olfactant recognition; odorant receptors have been referred to as “the jewel of olfactory research in the past 10 years” [33]. The MOE and VNO have some common features, but also significant differences in neuron types, primary structures of receptor proteins and signal transduction [33]. While the cloning experiments now rapidly advance our knowledge of receptor proteins, these important transmembrane biomolecules are currently less amenable to direct physical/biochemical studies that would lead to the elucidation of their interactions with pheromones.

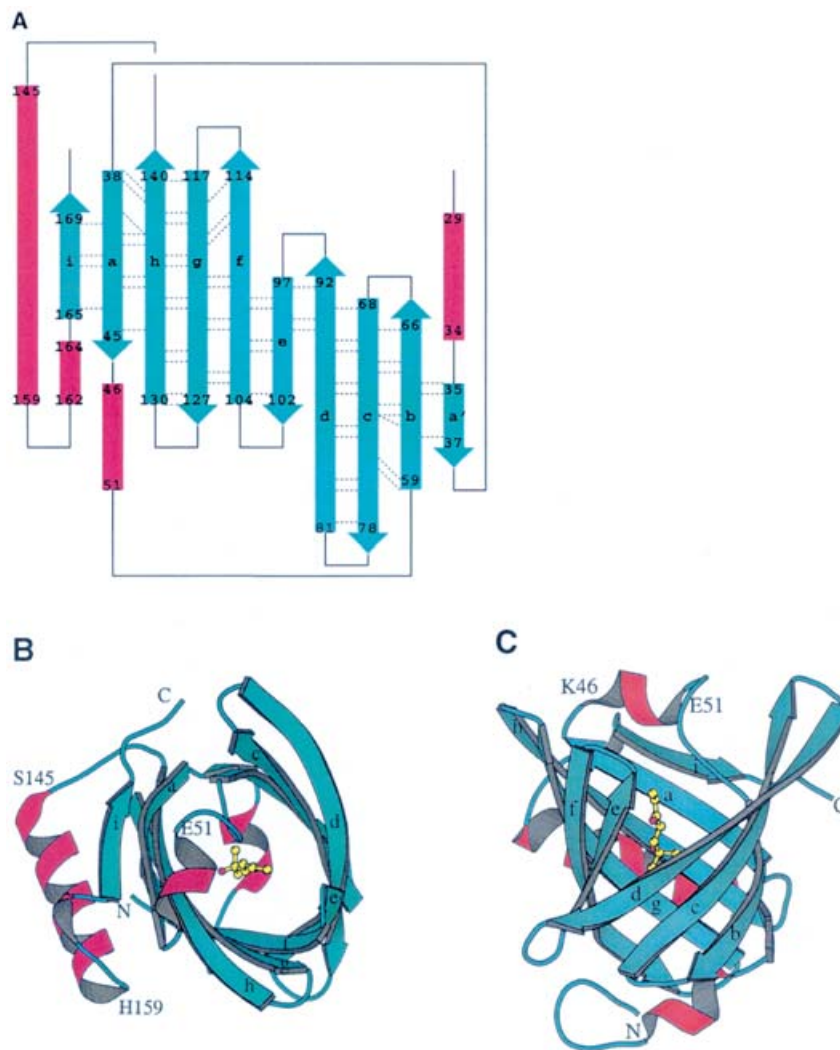
At present, it is considerably easier to study pheromone-protein interactions with a variety of soluble binding proteins of a smaller size. A number of such proteins may participate in the so-called perireceptor events [34] to transport pheromones, assist receptors, deactivate the reception site, etc. Interestingly, some lipocalins (carrier proteins) are known to be expressed in the nasal mucus of rodents, while other similar proteins are also excreted in the urine of mice (major urinary proteins; MUPs) [35] and rats (α 2u-globulins) [36], and the vaginal secretions of the golden hamster (aphrodisin) [37]. They all can bind small ligands, albeit in different tissues and different species, for different reasons.

Certain lipocalins have favourable features that facilitate their chemical/biochemical investigation: (i) easy crystallization for detailed structural studies of the sites of ligation [38]; (b) relatively small molecular size to study the relevant complexation phenomena (both dynamic and thermodynamic aspects) through NMR spectrometry [39,40] and biochemical calorimetry [41]; and (iii) availability in sufficient quantities for the investigation of structure and post-translational modifications. At present, we still have a very incomplete picture as to how this structural information pertains to the function of such proteins.

MUPs are excreted in the urine of laboratory and wild mice in a sexually dimorphic manner. As shown in ligand displacement studies, MUPs play a role in slow release of pheromones [42] and, more recently, their direct role in chemosignalling has also been proposed [43]. The recent results from our laboratory utilizing recombinant MUPs show significant variations in both the affinity and specificity of different isoforms for several mouse pheromones [41]. The differences measured between the MUPs excreted in urine and a variant expressed in the nasal cavity may shed some light on the structure-function relationships in release and capture of pheromones. It is encouraging that there are some methodologies at hand to investigate the fine details of pheromone-protein complex formation. As shown in Figure 2 and further described in a recent paper [38] using MUP-I and a male mouse pheromone as an example, the intimate details of the pheromone-binding sites can now become available through high-resolution crystallographic

Figure 2 | MUP-I structure

(A) A topology diagram illustrates the main chain hydrogen bond pattern (broken lines) between β -strands in the MUP-I β -barrel. (B) A ribbon diagram illustrates the position of 6-hydroxy-6-methyl-3-heptanone within the MUP-I pheromone-binding site and highlights the proximity of pheromone to the point of separation between β d and β e. (C) A ribbon diagram illustrates 6-hydroxy-6-methyl-3-heptanone binding viewed from the entrance to the β -barrel. Reproduced from [38] with permission from Cold Spring Harbor Laboratory Press.



studies. Moreover, the ligand orientation [39] and the dynamic motions within the protein–pheromone complex [40] were elucidated using NMR spectrometry. Related studies by isothermal titration calorimetry [41] show further details of the pheromone-binding phenomena.

Some protein structures encountered in chemical communication are glycosylated. Vomeronodulin, a putative pheromone transporter in the rat, is a 70-kDa glycoprotein with N-linked glycans [44]. A small glycoprotein with a sexually dimorphic expression and glycosylation (Y. Huang, Y. Mechref, J. Karty, J.P. Reilly, W. Ma and M.V. Novotny, unpublished work) has recently been isolated from the VNOs of male and female mice. Tentative evidence that the receptor proteins themselves are glycosylated structures raises the

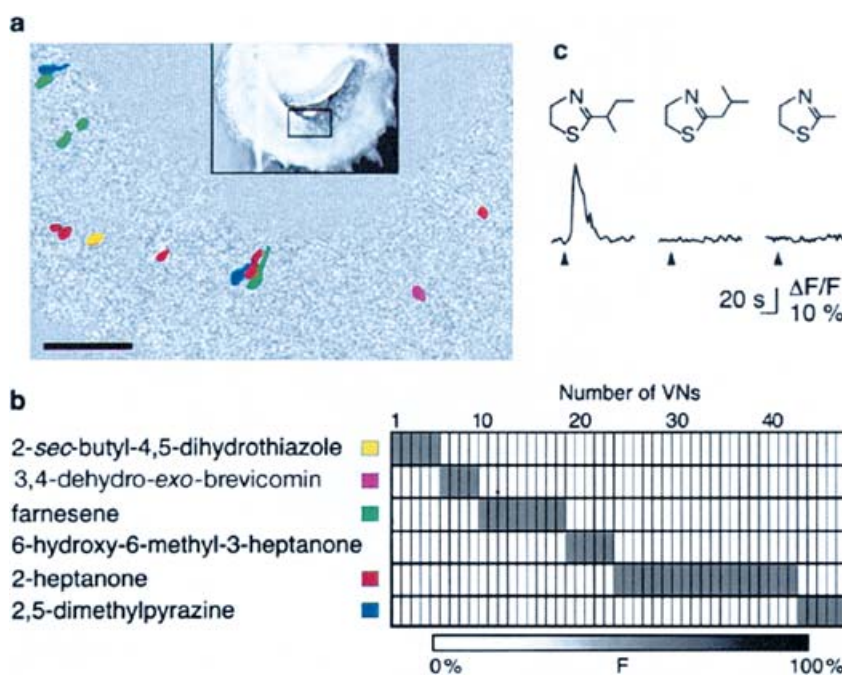
question of how important glycosylation is in mammalian chemical communication.

Research at the interface of chemistry and neurobiology

Using appropriate biological specimens from the VNO and the MOE, it is now rapidly becoming possible to follow transduction of pheromone signals into electrical signals that regulate hormonal and behavioural responses. Using electrophysiological techniques [7,8,45] and confocal calcium imaging, responses to mouse pheromones have been observed at amazingly low levels of detection (Figure 1) [7]. As shown in Figures 3(a) and 3(b) [7], responses of different

Figure 3 | Chemosensitivity of vomeronasal neurons

(a) Vomeronasal neuron activation map produced by successive stimulation with each of the six ligands listed in (b). (b) Summary of the tuning profiles of 47 neurons (out of approx. 1600 imaged cells). (c) Calcium imaging response to different thiazolines. Reproduced from [7] with permission from Nature Publishing Group.



neurons to successive stimulation with the same pheromones can be mapped. Figure 3(c) shows the remarkable selectivity to a chemical structure: from three related thiazole derivatives, only the natural pheromone, 2-sec-butyl-4,5-dihydrothiazole, elicits a response in this tissue slice. Unlike the results obtained with MOE using similar tissue preparations [46], VNO shows highly selective tuning properties irrespective of ligand concentrations.

There are many potential directions in using the neurobiological tools that are available at present. Confocal calcium imaging techniques can be set to investigate the effects of many other odorants, structural analogues, isomeric structures, etc. The effects of pheromone-binding proteins can be quantitatively evaluated. Through the use of known pharmacological agents, it will now be feasible to elucidate the entire cascade of signal transduction events. The use of mutant animals [47] will further help in unravelling the enormous complexity of chemosignalling events. As more pheromones become identified in species other than rodents, species-to-species differences in the use of chemical communication will be increasingly clarified.

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