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Olfactory mechanisms of stereotyped behavior: on the scent of specialized circuits

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Summary

Investigation of how specialized olfactory cues, such as pheromones, are detected has primarily focused on the function of receptor neurons within a subsystem of the nasal cavity, the vomeronasal organ (VNO). Behavioral analyses have long indicated that additional, non-VNO olfactory neurons are similarly necessary for pheromone detection; however the identity of these neurons has been a mystery. Recent molecular, behavioral, and genomic approaches have led to the identification of multiple atypical sensory circuits that display characteristics suggestive of a specialized function. This review focuses on these non-VNO receptors and neurons, and evaluates their potential for mediating stereotyped olfactory behavior in mammals.

Introduction

Olfaction powerfully instructs behavior, primarily through experiential association. For example, the smell of a previously unremarkable odor in a stressful environment is likely to initiate stress upon subsequent detection. In contrast, exceptional subsets of odorants have the unique capacity to generate behavior that is largely stereotyped both between individuals and upon repeated exposure (Table 1). While the stimuli that trigger most mammalian sensory systems are well defined, pheromones and those odors that instruct stereotyped behavior are largely unknown. This is due, in part, to the immense number of candidate odorants as well as technical limitations that have prohibited the use of traditional methods to de-orphanize mammalian odorant receptors [1]. Odorant receptors are located in the nasal cavity which is separated into distinct subsystems (Fig 1) that include the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). MOE neurons project their axons to particular neuropils (glomeruli) in the main olfactory bulb (MOB) with subsequent circuits primarily projecting to the olfactory cortex. In contrast, VNO neurons project to the accessory olfactory bulb (AOB) whose output neurons innervate the limbic system [2]. The clear anatomical segregation of these olfactory subsystems, and their expression of molecularly distinct populations of sensory neurons, led to the theory that they may each be specialized for differing function. Indeed, the MOE is established to sense odorants while the VNO has been found to detect pheromones [3]. Therefore, until recently, the search for chemical cues and receptors of specialized function, such as mediating species-specific behaviors, was primarily focused on the VNO. Interestingly however, genetic and behavioral studies have consistently indicated that VNO activity is not necessary for the display of some olfactory-mediated instinctual behavior [4] indicating the existence of uncharacterized, functionally specialized, behaviorally relevant olfactory sensory circuits outside the VNO. While the investigation of VNO circuits that influence behavior

remains of great interest (see Box 1), recent studies have now identified neurons in the MOE and additional (non-VNO) olfactory sub-systems that display characteristics consistent with the detection of specialized odorants and the ability to mediate stereotyped behavior. Molecular and functional identification of these neurons has the potential to determine the rationale of multiple olfactory subsystems, the range of olfactory coding strategies, and provide molecular tools to determine if olfactory specializations function in humans.

Pheromone detection, beyond the VNO?

Classic studies showing the attractive response of the sow to androstenone (naturally released by boars) as well as the robust suckling behavior of the European Hare [5-6] have directly implicated the MOE in mediating pheromone-evoked responses. Functional experiments indicate that in both instances behavioral responses are VNO independent [5,7-8]. More recently, deletions of CNGA2 and ACIII, essential components of the canonical MOE signaling pathway, as well as complementary deletions in TrpC2, the primary sensory channel of the VNO, revealed that neurons outside the VNO are sufficient to display suckling and mating behavior and necessary to generate male-male aggression [9-13]. Whether these findings are due to the specific deletion of specialized signaling circuits or an indirect result of catastrophic olfactory loss is unknown. It has been suggested that anosmic mice either reduce sniffing which inhibits VNO function, or enter a severely depressed motivational state thereby secondarily impairing all social behavior [9,14]. This was addressed recently by engineering a mouse with over 95% of the olfactory sensory neurons expressing the same odorant receptor (M71) [15•]. These mice do display impaired olfactory discrimination but can still detect a surprising range of odorants, presumably utilizing the remaining 5% of OSNs that express a natural variety of receptors. This residual olfactory capacity relieves concerns about secondary motivational defects or general VNO function. Interestingly, these mice do not display appropriate stereotyped mating behavior, which further strengthens the hypothesis that, like VNO neurons, MOE neurons similarly mediate social behaviors. In a system that has over 1200 distinct sensory neuron types how can the relevant specialized subset be identified? Investigation has focused on neurons whose cell bodies or axonal projections are anatomically segregated from canonical MOE neurons as well as those that express novel sensory transduction molecules (Fig. 2).

Specialized sub-systems, specialized functions?

Recently a group of morphologically distinct neurons, whose cell bodies reside outside of the MOE, have been implicated in pheromone-mediated behavior. The Grueneberg ganglion (GG) was first thought to represent a branch of the terminal nerve until its rediscovery as an olfactory sub-system in 2005 [16-18]. GG neurons are present at birth, found in many mammalian species, and project to a “hemi-necklace” of glomeruli at the very caudal MOB (Fig. 1,2A) [18] a region thought to be activated during nursing [19-20], collectively leading to hypotheses that they detect suckling pheromones [17]. However, lesioning the GG axon tract has no effect on nursing behavior [21••]. Instead, two distinct functions have recently been described. Mamasuew *et al* found that a subset of GG neurons of neonatal mice induce cFos in response to cool ambient temperatures, thereby acting as a thermosensor [22]. Interestingly, this may achieve the goal of a maternal pheromone, attracting a pup to the warmth of its mother, using an alternative sensory modality.

Independently, Brechbühl *et al* monitored intracellular calcium concentrations in individual GG neurons when exposed to urine, milk, mammary secretions, CO_2 or temperature change, but observed no responses. However they did find that critically stressed mice release a signal that activates over 95% of the assayed GG neurons [21••]. The mechanism of this robust response is unclear considering that the molecular profile of GG neurons is not homogeneous.

Indeed the GG has been shown to express components common with both VNO neurons (receptor V2R83b, Gαi2, Gαo) and canonical MOE neurons (olfactory marker protein, ACIII, Gαolf, and odorant receptors) [23]. Some neurons express the membrane receptor guanylyl cyclase sub-type, GC-G [24] while others alternately express GC-A [25], neither of which are found in the MOE or VNO. Importantly, behavioral analysis revealed strong freezing response to the stress-emitted signal, which is abolished upon neonatal GG lesion. The authors conclude that the GG mediates an innate response to an alarm pheromone emitted from the stressed mice [21••]. Identification of this stress signal and its sensory receptor(s) will provide the tools to confirm this function. If the GG is validated to detect pheromones it reinforces conclusions drawn from VNO analysis: that the organization of the nasal cavity into distinct subsystems may serve to generate specialized olfactory function (Fig 2A).

Necklace MOE neurons defy conventional mechanisms

Classical anatomical studies have identified another morphologically distinct set of glomeruli embedded in the MOB. These specialized “necklace” glomeruli, named because they encircle the caudal olfactory bulb like beads on a string [26], appear to be adjacent to, but different from the “hemi-necklace” of the GG neurons (Fig. 1)[18]. The necklace glomeruli are innervated by molecularly atypical neurons that do not express the canonical cAMP signaling proteins that define MOE neurons and instead express components of a cGMP signaling cascade [27-28]. No GPCRs have been identified in these neurons; however a membrane receptor guanylyl cyclase (GC-D) has been shown to be necessary for function [29•].

Early experiments associated the necklace region with suckling [19-20], but a series of recent independent studies came to alternate, unexpected conclusions. Utilizing electrophysiological techniques, one group found that GC-D neurons detect two natriuretic peptides, uroguanylin and guanylin [29•], at least one of which is present in mouse urine (a rich source of known VNO-mediated pheromones). Heterologous expression found GC-D to be activated by uroguanylin, but not guanylin [30]. Neurons from GC-D mutant mice fail to respond to natriuretic peptides indicating a primary role for GC-D in signaling. It has been reported that sub-sets of GC-D expressing neurons differentially detect guanylin, uroguanylin, or both [29•], however the mechanism underlying peptide specificity is unclear given that no other membrane receptor has been identified in these neurons. Both peptides are involved in regulating electrolyte balance suggesting the possibility that necklace neurons monitor metabolic status. Another elegant series of studies reported that the same neurons express a carbonic anhydrase that converts carbon dioxide to bicarbonate [31•]; this directly activates the cyclase domain of GC-D and triggers neural activation [32•-33]. Near atmospheric concentrations of CO_2 delivered to anesthetized mice specifically induced activity from mitral cells that innervate necklace glomeruli, establishing the beginnings of a functional circuit [31•].

The importance of CO_2 and uroguanylin in olfaction is unclear as both are emitted from other animals as well as endogenously present in the nasal cavity. It has been observed that naris occlusion diminishes tyrosine hydroxylase activity throughout the MOB, including the necklace, suggesting either a role in detecting external stimuli or the requirement of active respiration [34]. It remains of great interest to determine if the molecular and anatomical distinction of necklace neurons generates a specialized behavior, as no deficits were identified in GC-D knockout mice [29•]. Interestingly, while all other studied MOB glomeruli are exclusively innervated by neurons expressing the same GPCR [35], necklace glomeruli defy this convention. Instead they contain mixed sensory input both from GC-D neurons as well as canonical MOE neurons [36]. It is tempting to speculate that this unusual heterogeneous composition of necklace glomeruli may provide a mechanism for signals from odorants to be integrated with stimuli that encode specialized functions.

Olfactory receptors revisited

The hypothesis that olfactory functional specialization may be achieved through molecular specification prompted the search for additional atypical classes of MOE neurons. Liberles and Buck reasoned that pheromone sensing neurons of the MOE may express a GPCR of a different sub-family from those expressed by canonical neurons. Through genomics and expression analysis they identified a small population of MOE neurons that express trace amine-associated receptors (TAARs) [37••]. Importantly, each individual neuron expresses only one TAAR and displays no co-incidental expression with canonical olfactory receptors, suggesting the TAAR molecularly defines a specific sensory circuit. Moreover, the approximately 90:1 ratio of canonical receptors to TAARs suggests that they may indeed serve a highly specialized function in the MOE. Heterologous expression of TAARs enabled the functional screening of odorants to find that they selectively bind volatile amines. These amines may convey social information as at least one is naturally enriched in the urine of stressed rodents and humans [37••]. At this time, the functional importance of TAAR expressing neurons has not yet been established but their characteristics are consistent with candidate pheromone detectors [38]. Whether the glomeruli innervated by TAAR neurons project to an anatomically distinct region of the MOB, similar to the necklace (Fig 2B), remains to be determined. Interestingly, volatile amines are odorous and therefore may also be detected by ensembles of canonical olfactory receptors. If so, it will be important to determine whether amine odor activity generated from canonical MOE and TAAR neurons is differentially processed in the brain.

Spatial organization, zones of innate behavior?

An alternate mechanism to specialize olfactory function may not depend on molecular or anatomical characteristics of sensory neurons, but instead may occur through synaptic distinctions (Fig 2C). Both the Dulac and the Buck labs have shown by independent methods that subsets of MOE neurons establish primary circuits to GnRH-expressing neurons in the hypothalamus, confirming the occurrence of such post-synaptic specialization [39-40]. Recently Sakano and colleagues created genetic tools to selectively ablate large ensembles of either dorsal or ventral glomeruli, generating a platform to test the biological significance of each MOB domain [41••]. They analyzed the ability of these mutants to detect and behaviorally respond to a series of innately aversive odorants including 2-methylbutyric acid, the odor of spoiled food, or trimethyl-thiazoline, a pungent odor secreted by foxes. Interestingly, mice missing dorsal glomeruli lack both innate aversion and a characteristic stress response to both odors. Strikingly, the mutant mice could still detect these odors, discriminate them from other odorants and be conditioned to avoid them through the remaining ventral glomerui. The authors conclude that a number of glomeruli in the dorsal zone are necessary for the innate aversive response, while ventral glomeruli are sufficient for olfactory conditioning (Fig 2C) [41••]. Further screening of these mutants with a larger panel of stereotypically aversive odors will help determine if a general feature of the dorsal MOB is to generate innate behavior. If so, it will be of interest to determine if behavior is mediated by canonical neurons or by molecularly specialized neurons within the zone.

Molecular evaluation of human pheromone detection

Human pheromomes are the subject of intense popular fascination, but have yet to be definitively identified. Their study is particularly challenging, not least because experimentally controlling the identification of learned associative responses from instinctive responses is nearly impossible using human subjects. A functional VNO is likely absent in humans and key VNO-specific genes have been demonstrated to be pseudogenized in our lineage [42]. Like the VNO, the GG has been identified only in human embryos and likely regresses during fetal development [16,21]. GC-D is also pseudogenized in primates, and human necklace glomeruli

have not been described [43]. In contrast, TAAR receptors persist in the genome of many diverse vertebrate species, including humans, and are widely expressed in the olfactory neurons of bony fish [44-45], however it is currently unknown whether TAARs are expressed in human olfactory neurons.

Perhaps the strongest candidate for human pheromone status is androstadienone, a component of male sweat that is reported to maintain cortisol levels in women and influences their attraction to men in some contexts [46-47]. The human olfactory receptor for androstadienone was recently identified as OR7D4 and found to have naturally occurring sequence variants that correlate with both *in vitro* activation of the receptor and the pleasantness of androstadienone perception [48••]. Interestingly this receptor is a canonical olfactory GPCR, with no obvious signs of molecular specialization for pheromone detection. Therefore if androstadienone is a human pheromone, it appears likely that the organization of its glomeruli or post-synaptic projections will be what distinguishes its sensory neurons from those responding to other odorants.

Though there have been many recent advances, the identification of cognate ensembles of ligands, receptors, circuits and behavior will ultimately be required to determine the general mechanisms that initiate stereotyped olfactory-mediate behavior in mammals.

Box 1. VNO ligands and receptors

Recently there have been exciting advances towards identifying the molecules and mechanisms that underpin VNO function. Functional screening has revealed over 50 new ligands with pheromone characteristics. Two large clusters of genes, the Major urinary proteins (Mups) and exocrine-gland-secreting peptides (ESPs), have been shown to encode secreted ligands [51-52]. Variants of both show sexually-dimorphic expression, activate VNO sensory neurons of the mouse [50,53] and, in the case of Mups, elicit stereotyped male aggressive behavior [50]. Additionally, a new class of metabolite, sulfated steroids, was found to be sexually dimorphic, expressed in female mouse urine, and to robustly activate VNO neurons [54]. Strikingly, 80% of the VNO neural response to female urine is lost by sulfatase treatment supporting their importance as olfactory ligands [54]. Sulfated steroids have been shown to function as a migratory pheromone in lamprey [55]. Their function in mice is unknown but they are hypothesized to provide information about gender and physiology, such as recent stress [54].

Parallel discoveries have advanced our understanding of VNO sensory detection. A third receptor family, the formyl peptide receptor-like (FPR) proteins, was discovered in the VNO [56•-57•]. Though their function has not been established, they are activated by modified peptides characteristically produced by bacteria [57•]. Therefore it has been proposed that FPR-expressing neurons may detect disease state in other animals. Finally, stimulation with major histocompatibility complex (MHC) peptides provided insight into mechanistic logic of VNO neuron activation. Remarkably, only a subset of neurons expressing a labeled VNO receptor (V1Rb2) responded to a purified MHC stimulus, suggesting that this receptor may not be sufficient to determine ligand response [58].

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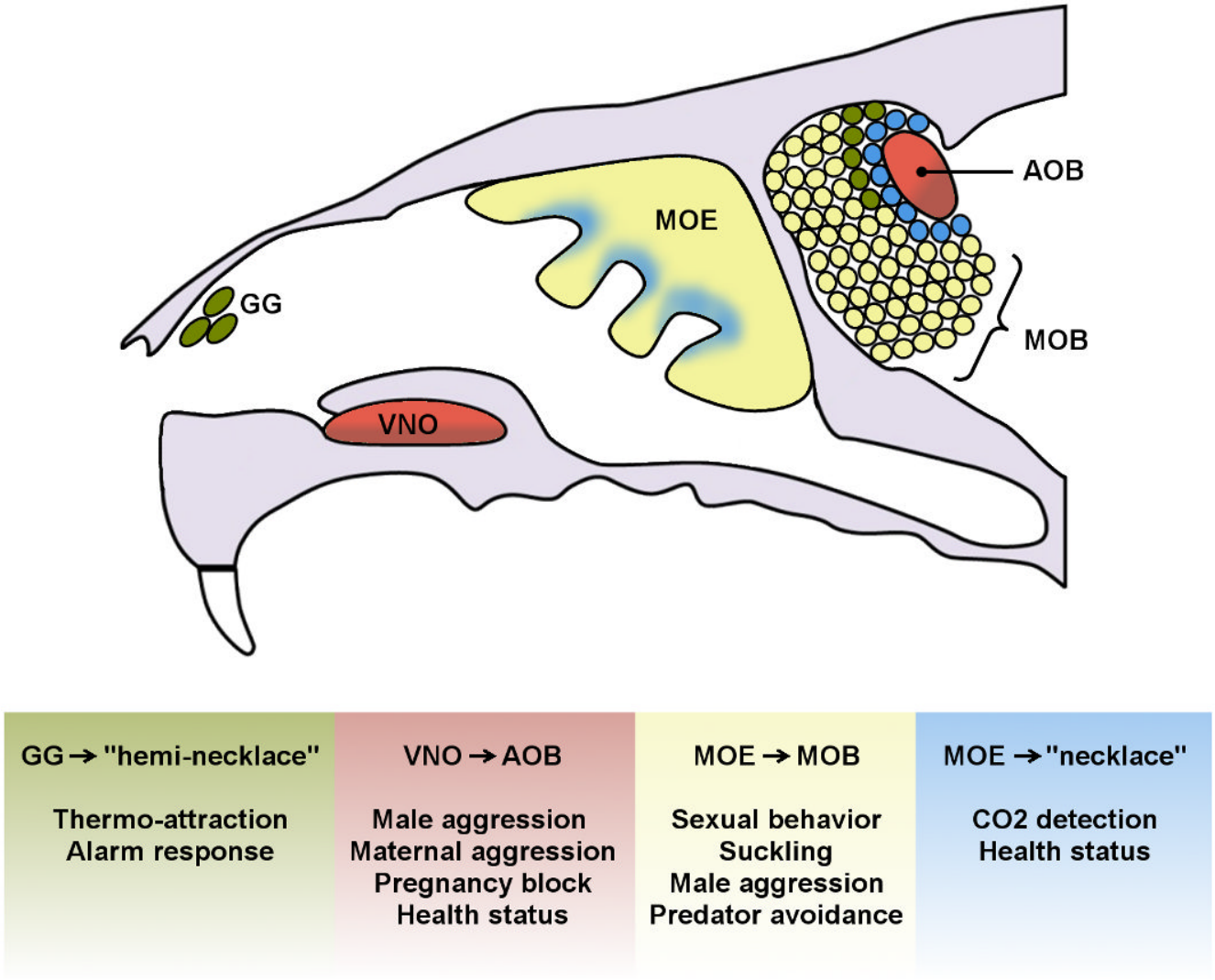

Figure 1. Olfactory sub-systems implicated in mediating innate behavior

Upper: Organization of olfactory subsystems in the mouse; vomeronasal organ neurons (VNO) innervate the accessory olfactory bulb (AOB), canonical main olfactory epithelium (MOE) neurons project axons to the main olfactory bulb (MOB, yellow), Gruenberg ganglion (GG) neurons project to the MOB hemi-necklace (green), and molecularly specialized MOE neurons project to the MOB necklace (blue). Lower: Innate, pheromone, or specialized olfactory responses reported to be mediated through each sub-system.

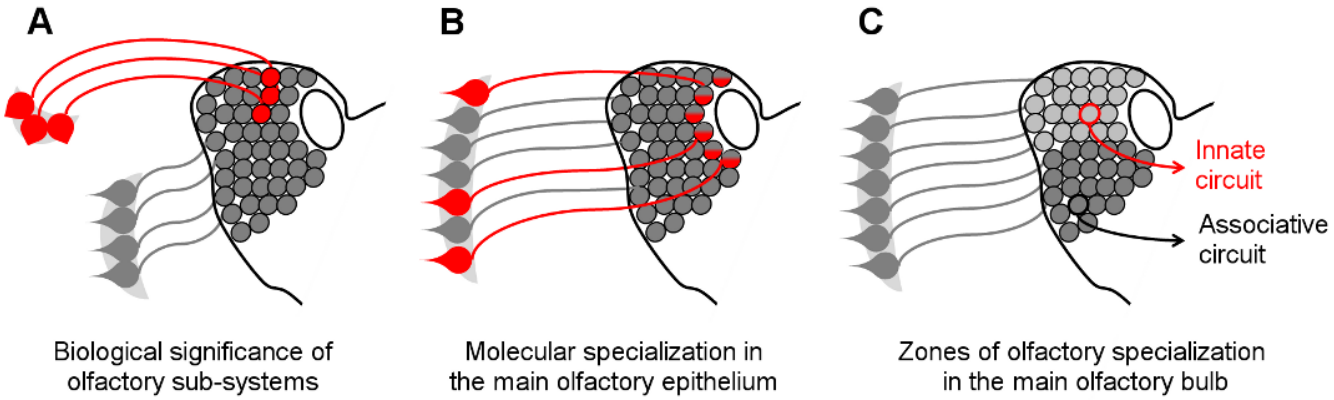

Figure 2. Mechanisms encoding innate behavior: beyond the vomeronasal organ

Multiple mechanisms have been postulated to encode innate olfactory information. Three current hypotheses: A) Anatomical segregation of sensory neurons distinguishes specialized processing (red) from general odor detection (grey). The VNO and GG (represented) are being investigated to support this mechanism. B) Molecular specialization of MOE neurons may enable pheromone detection (red). GC-D (represented) or TAAR expressing neurons may utilize this mechanism. C) The MOB glomeruli may be organized in functional zones. Analysis of mice lacking the dorsal zone (shaded light) suggests its necessity for innate coding.

Table 1
Classification of olfactory cues by behavioral significance

	Pheromone	Instinctive cue	Odorant
Definition	Social cues emitted by one member of a species that provoke a specific behavioral or physiological response when detected by another member of the same species [49].	Chemical cues that initiate a conserved, stereotyped behavior on first and subsequent exposures.	Chemicals that encode a contextually dependent perceptual quality and can trigger an associative or learned behavior with appropriate training.
Example	Major urinary proteins (Mups) found in the urine of adult male mice initiate aggressive behavior in rival males [50].	Spoiled food odors elicit avoidance in rodents [41••].	Mice rapidly learn odors that are paired with a reward, and will prefer that odor on subsequent exposure [15•,41••].