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Sexual dimorphism in olfactory signaling

Lisa Stowers^a and Darren W Logan^b

^aDepartment of Cell Biology, The Scripps Research Institute, ICND222, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

^bWellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1HH, UK

Abstract

What makes males and females behave differently? While genetic master-regulators commonly underlie physical differences, sexually dimorphic behavior is additionally influenced by sensory input such as olfactory cues. Olfaction requires both ligands for signaling and sensory neural circuits for detection. Specialized subsets of each interact to generate gender-dimorphic behavior. It has long been accepted that males and females emit sex-specific odor compounds that function as pheromones to promote stereotypic behavior. Significant advances have now been made in purifying and isolating several of these sex-specific olfactory ligands. In contrast, the neural mechanisms that enable a gender dimorphic response to these odors remain largely unknown. However, first progress has been made in identifying components of sexually dimorphic olfactory circuits in both *Drosophila* and the mouse.

Introduction

Males and females display well characterized behaviors specific to each gender such as territorial aggression, parental care, and mating. While genetic factors have been shown to underlie sex-specific anatomy and physiology less is known about neural mechanisms that control behavioral dimorphisms. Sex-specific behavior is primarily released in response to specific sensory information. Such stimuli include coloration, mating songs, and chemosensory cues, such as pheromones [1]. In many species, including the fly and the mouse, olfactory sensation is necessary to display appropriate sex-specific behavior. Loss of olfactory sensory transduction impairs, ablates, or transforms the appropriate display of gender-specific behavior [2-8]. How olfaction regulates specific behaviors is mostly unknown. In the mouse, each olfactory neuron expresses one of approximately 1300 G-protein coupled receptors (GPCRs) which can be located in one of several anatomically distinct subsystems [9-11]. Furthermore, much of the olfactory system appears to function similarly in both males and females, such as the sensory response initiated by food odors. Amid such complexity, the ability to molecularly identify and study the specific olfactory sensory neurons and circuits that underlie gender-dimorphic behavior has been daunting. However, recent advances in the purification of signaling ligands, primarily emitted from either males or females, has now led to the identification of their cognate subsets of sensory neurons and enabled the study of the neural mechanisms initiate sex-specific behavior.

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Sexually dimorphic olfactory ligands

A number of species produce a range of gender-specific chemicals which are transmitted to others by secretion in exocrine fluids such as urine, saliva, tears, or sweat. In the mouse, sex-specific ligands have primarily been identified by comparing the bioactivity and odor constituents of male and female secretions. The vomeronasal organ (VNO) is a specialized olfactory subsystem that has been well characterized to detect these ligands [12-14•]. A variety of electrophysiological and imaging approaches show approximately 20-50% of sensory neurons in the VNO to be differentially activated by male and female urine [12-13]. The use of differential VNO activity as an assay to purify sex-specific ligands has enabled the identification of a large family of sulfated steroids in the urine of female mice. Purification of these bioactive compounds identified 13 distinct sulfated compounds in female urine that are undetectable in male urine [15-16] and female urine treated with sulfatase lost over 80% of its VNO stimulating activity. When a panel of 31 synthetic sulfated compounds was evaluated for the ability to be detected by the VNO, the aggregate response was similar between male and female neurons [15], suggesting both sexes can equally detect sulfated steroids. This does not preclude sex-differences in the detection of specific compounds however, as different VNO neurons are tuned to respond to different sulfated steroids [17] and awaits further study. Currently the type of information transmitted by urinary sulfated steroids is unclear; though at least two compounds are more abundant in stressed females, and therefore they could function as honest signals of both sex and physiological condition [15-16].

In the mouse, the emission of male-specific odors is testosterone dependent, either by transcriptional regulation, as a chemical derivative, or a metabolic byproduct of an androgen. Known male-specific signals primarily evoke two divergent behaviors among conspecifics: attraction or sexual receptivity in females and/or aggression in males. Direct comparison of bioactivity from intact and castrated males identified major urinary proteins (Mups) as male-specific olfactory signals. Mups are encoded by a highly homologous family of androgen-regulated genes that are abundantly expressed in the liver, resulting in protein excreted in male mouse urine [18-20]. Purified Mups are detected by VNO sensory neurons and promote territorial aggression when detected by a rival male [21]. Interestingly, when detected by females Mups do not initiate aggression. Instead, at least one Mup is responsible for attracting wild-derived female mice to male urinary scent marks [22], presumably to promote mating. Urine from males that lack expression of this particular Mup is not attractive to females [22], demonstrating both the specificity and necessity of the sense of smell in mediating this behavioral response.

In mice there are other gene-families that encode sex-specific protein pheromones. Among these are the exocrine-secreted peptides (ESPs) and androgen-binding proteins (ABPs) [23]. While the significance of ABPs remains to be determined, several ESPs have been well characterized. ESP36 is secreted into the tears of juveniles and adult female mice, but not males where its expression is down-regulated by testosterone [24]. However it is yet to be determined whether ESP36 has a role in pheromone communication. Like Mups, ESP1 expression is male-specific. It is secreted into the tears, providing accessibility to other mice engaging in chemo-investigation [24-25]. Purified ESP1 is detected by VNO neurons that activate neural circuits which generate a female-specific mating stance. Recently, Haga *et al.* have engineered mice specifically lacking ESP1 sensory receptors [26••]. Remarkably, even though all other sensory systems (including most of the olfactory system) are fully functional in these mutants, females rarely adopt a mating stance in response to males. This demonstrates the necessity and specificity of ESP1 as an olfactory cue to mediate sex-specific behavior.

Sexually dimorphic olfactory circuits

What are the neural mechanisms that enable males and females to behave differently in the presence of the same olfactory ligand? One model predicts that the sensory neurons of males and females differentially respond to the same cue. In the mouse, intense investigation has revealed VNO sensory neurons that are tuned to either male or female cues [12-14•,27]. VNO neurons of mice are thought to detect individual odor ligands by specifically expressing one variant of >350 GPCRs [9-11]. Dimorphic behavioral responses could be initiated to the same ligands by absolute or differential gender-specific expression of subsets of receptors [28]. However, stimulation with gender-specific ligands has not found significant qualitative or quantitative differences in the activation of male or female VNO neurons [12-14•,27]. The generation of mice with fluorescent ESP1 receptor neurons now enables comparison of the identical neural circuit between males and females exposed to ESP1 [26••]. As previously shown with other VNO receptors of unknown function [29], no significant quantitative differences between male and female VNO sensory neurons or their patterns of projections into the brain were identified, suggesting both sexes equally detect and transmit the pheromone signal through this level of processing (Figure 1a). However, a survey of immediate-early gene (cFos) induction patterns in sub-cortical brain regions following ESP1 exposure showed a range of sexual dimorphisms in neural activation. For example, ESP1 induced increases in cFos in the female ventromedial hypothalamus, while in the male the medial preoptic area showed enhanced neural activation (Figure 1a) [26••]. This differential sensory processing may account for the sex-specificity of the pheromone-mediated behavior. However, a different mechanism of sex-specific regulation emerged from studies on another strain of mouse in which males show no cFos induction in the VNO following exposure to ESP1 [25]. Correspondingly, these non-responsive males secrete ESP1 in their tears whereas, like females, responsive males do not [26••]. This prompted the authors to propose that the sensory neurons of males that produce ESP1 fail to respond through ligand desensitization. Given that wild-derived male mice do appear to express high levels of ESP1 [26••], signal-mediated desensitization could play a prominent role in modulating dimorphic responses to sex-specific pheromones.

Within the brain, gender-specific behavior may be achieved by the development of distinct neural circuits in each sex. Genetic mutants that lack the primary sensory transduction channel of the VNO, TrpC2, are unable to sense the odor ligands that initiate appropriate gender-specific behavior [2-4,21]. While female TrpC2 mutants display gender appropriate physiological characteristics, such as a normal estrus cycle and fertility, they do not display female typical behaviors such as maternal aggression and nest building. Instead, both the mutants, and adult females that have had their VNO surgically ablated, display male-typical sexual and courtship behavior [3••]. The authors conclude that the female brain harbors circuits for both male and female-typical behavior and environmental modulators, such as a functional VNO olfactory system, are necessary to initiate the activity of the gender-appropriate circuit [3••]. Elegant experiments from the Shah lab have now begun to identify central mechanisms that may account for such a gender-dimorphic sensory modulation of behavior. Using genetically engineered mice, they find that testosterone produced just after birth in males, masculinizes the brain through its conversion by aromatase to estrogen [30•]. Females do not normally produce estrogen during brain development, but post-natal supplements are sufficient to masculinize their brains and promote some male-typical behaviors such as territorial aggression [30•]. Males with a brain specific deletion of the androgen receptor do display male-specific behaviors however at a reduced probability [31-32]. Other studies have linked the expression of sex hormones with histone modifications that lead to epigenetic sex-specific gene silencing [33], an effect which may be imprinted and underlie the development of sex-specific circuits [34-35]. How neurons expressing aromatase and androgen receptor generate sex-specific function is not

understood; but when considered with analyses of TrpC2 mutant behavior, these hormonal-responsive neurons may dictate the sexual ‘state’ of the individual to activate a gender-appropriate circuit.

***Drosophila*: from odor ligands to sex-specific behavior**

Significant advances have been made towards the anatomic and genetic characterization of a sexually dimorphic olfactory circuit in *Drosophila*. Like mammals, fruit flies transmit social information via pheromones. One such chemical specifically expressed on the cuticle of male fruit-flies, 11-cis-vaccenyl acetate (cVA), is known to evoke different behaviors when detected by either sex. In males it suppresses courtship behavior at high concentrations and initiates male-male aggression at lower concentrations, whereas in females it promotes courtship behavior [7•-8•]. It has also been shown to promote aggregation in both sexes under some conditions [36-37].

The pheromone, via an intermediary odorant binding protein, LUSH, activates receptor Or67d expressing olfactory sensory neurons in the fly’s antenna [38]. Electrical recordings from male and female Or67d expressing OSNs found no difference in response to cVA, suggesting they are equally sensitive to pheromone detection, and mutant flies of both sexes that lack Or67d function show deficits in their respective responses [7•-8•]. Moreover, the correct dimorphic behaviors could be restored in either sex by expressing a moth pheromone receptor in place of Or67d and presenting the flies with its corresponding ligand, elegantly demonstrating the sufficiency of these neurons for mediating cVA-evoked responses in both males and female flies [8•].

fruitless, a transcription factor that has male- and female-specific isoforms has been found to underlie the generation of sex-specific neural circuits. The male form (Fru^M) is expressed in about 2000 neurons distributed throughout the male brain, and is both necessary and sufficient for male behaviors [39]. Targeting a GAL4 transactivator to the *fruitless* locus enabled the visualization of Fru^M-expressing neurons in males, and the equivalent neurons in females [40]. The Or67d-expressing neurons are among a small proportion of olfactory neurons that are positive for Fru^M in males, but not in females (Figure 1b).

Or67d sensory neurons project to a single glomerulus in the antennal lobe, DA1, where they synapse with second-order projection neurons (PNs). Remarkably, Fru^M-expressing PNs appeared to synapse specifically with Or67d-expressing neurons, suggesting that an entire neural circuit may be molecularly defined by a male-specific Fru^M isoform (Figure 1b) [40]. However, apart from a dimorphism in the size of the DA1 glomerulus (it is slightly larger in males than in females [40-41]) it remained unclear until recently how Fru^M might alter the male circuit to mediate a different behavioral response to cVA from the female.

Neural tracing studies in flies expressing dynamic fluorescent proteins in the Fru^M-expressing PNs revealed no qualitative difference in innervations of the DA1 glomerulus in both sexes and recorded no electrophysiological dimorphisms after stimulating with the pheromone *in vivo* [42••]. This implies that the size difference in the DA1 glomerulus does not significantly influence the post-synaptic signal and thus cannot account for the sex-specific behavioral response. To determine where the signals diverged in the brain, the projection patterns of the glomerular output neurons (PNs) were analyzed in both sexes, and mapped on to a reference fly brain for direct spatial comparison [43]. PNs from the male DA1 have additional axon branches that are singularly and reproducibly missing in females [42••]. These dimorphic branches extend ventromedially in the lateral horn (Figure 1b), overlapping with a region of the brain that is known to be larger in males than in females [43], and are not seen in other male PNs that do not express Fru^M. The patterns of projections of PNs of *fru* mutant males showed a significant reduction in the dimorphic

arbors, indicating that the dimorphic projection is a consequence of Fru^M. Finally, expression of Fru^M in female flies generates the male-specific projections and the production of courtship behaviors similar to males [39,42••]. The extent to which different patterns of PN arborization ultimately generate behavioral dimorphism remains to be determined, as another *Drosophila* sensory circuit generates sex-specific olfactory behavior without expressing Fru^M or obviously displaying dimorphic PN arbors [44].

These recent advances in identification of specialized olfactory ligands now provide the tools to activate, identify, and study the neural mechanisms that underlie basic behavioral differences between males and females.

Box 1: Olfactory dimorphism in humans

Studies into human olfactory dimorphism have largely focused on the physiological and psychological effects of odorous sex-specific steroids including 14,16- androstadien-3-one (AND), a derivative of testosterone enriched in male sweat, saliva and semen. AND is structurally related to androstenone, a pheromone naturally produced by boars that stimulates sexual-receptivity in female pigs, and thus has been proposed as a candidate human pheromone [45]. Indeed, when sniffed by human volunteers synthetic AND enhanced positive mood state and autonomic arousal in women but not in men [45-46]. Curiously however, it was subsequently observed that the positive effect on female mood occurred only when the scientist applying the stimulus was male, suggesting the importance of environmental context in releasing the emotional response [47-48]. There is further evidence that AND activates a dimorphic olfactory circuit in the receiving individual. Position emission tomography (PET) of heterosexual males and females exposed to either AND or EST (estra-1,3,5(10),16-tetraen-3-ol, an estrogen-derived compound postulated to be a putative female-derived pheromone) revealed striking sex-specific, reciprocal patterns of brain activity. In females AND robustly stimulated blood flow to the hypothalamus while in males an overlapping hypothalamic region was activated by EST [49]. When these experiments were repeated in homosexual men and women, the hypothalamic activation patterns reversed similar to the patterns from heterosexual members of the opposite sex; therefore this dimorphism is congruent with sexual-orientation rather than genetic sex [50-51]. Importantly, androstenol is the only other odorant that has been observed to have hypothalamic-stimulating properties in either sex, indicating specialism in the dimorphic detection circuitry of these odors [52].

While these studies provided intriguing, albeit indirect, evidence of an olfactory-mediated influence on gender dimorphic behavior in humans, a direct impact on the endocrine system was reported recently for AND. Wyart and colleagues measured cortisol levels in the saliva of heterosexual women after sniffing AND or a control odor (both provided by a male experimenter) [53]. Cortisol secretion is stimulated by the hypothalamic production of corticotrophin-releasing hormone and has been shown to correlate with arousal and mood. Sniffing AND maintained significantly higher levels of cortisol than controls, in addition to enhancing mood, sexual and physiological arousal [53].

Occluding the human VNO does not impair the percept or brain activity in response to AND, suggesting detection occurs by the main olfactory system [54]. Consistent with this, a molecular approach to study the mechanism of AND detection identified a human odorant receptor, OR7D4, that is selectively activated by AND and androstenone [55]. Olfactory testing followed by genotyping identified an OR7D4 sequence variant in the human population that had a strong phenotypic correlation with the intensity and hedonic perception of AND. Expression of the variant *in vitro*, showed a significant impairment of AND sensitivity [55]. The identification of a functional olfactory receptor for AND

will permit the control of OR7D4 genotype in future cohorts for PET scanning, behavioral or neuroendocrine responses studies, and enable the use of animal models to determine whether AND/androstene signaling is an evolutionary conserved dimorphic circuit in mammals.

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Figure 1. Sexual dimorphism in olfactory signaling

Recent experiments have revealed mechanisms for generating gender-specific behaviors in mice and *Drosophila* [26••,42••]. (a) ESP1 evokes a mating stance when detected by Trpc2- and VR2p5-expressing VNO neurons of female mice (top). Although males have the same neurons that project to the same glomeruli (bottom), there are gender differences in neural activity in central brain regions (green arrows) in response to ESP1, presumably resulting in the sex-specific behavior. Additionally, in strains of male mice that produce ESP1 endogenously, signal-mediated desensitization may occur (gray line, bottom). BST, bed nucleus of the stria terminalis; Me, medial amygdaloid nucleus; PMCo, posteromedial cortical amygdaloid nucleus; MPA, medial preoptic area; VMH, ventromedial hypothalamus. (b) 11-cis-vaccenyl acetate (cVA), a male-specific *Drosophila* pheromone, evokes different behaviors when detected by Or67d-expressing olfactory neurons of either sex. In males (bottom), the circuit is patterned by the male-specific isoform of *fruitless* (*Fru^M*), the DA1 glomerulus is larger than in females, and the projection neurons have a male-specific arbor (purple).