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Sexual differentiation of pheromone processing: Links to male-typical mating behavior and partner preference

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Abstract

Phoenix et al. (Phoenix et al., 1959) were the first to propose an essential role of fetal testosterone exposure in the sexual differentiation of the capacity of mammals to display male-typical mating behavior. In one experiment control male and female guinea pigs as well as females given fetal testosterone actually showed equivalent levels of mounting behavior when gonadectomized and given ovarian steroids prior to adult tests with a stimulus female. This finding is discussed in the context of a recent, high-profile paper by Kimchi and co-workers (Kimchi et al., 2007) arguing that female rodents possess the circuits that control the expression of male-typical mating behavior and that their function is normally suppressed in this sex by pheromonal inputs that are processed via the vomeronasal organ (VNO)—accessory olfactory nervous system. In another Phoenix et al. experiment, significantly more mounting behavior was observed in male guinea pigs and in females given fetal testosterone than in control females following adult gonadectomy and treatment with testosterone. Literature is reviewed that attempts to link sex differences in the anatomy and function of the accessory versus the main olfactory projections to the amygdala and hypothalamus to parallel sex differences in courtship behaviors, including sex partner preference, as well as the capacity to display mounting behavior.

Keywords

partner preference; vomeronasal organ; olfactory sensory neurons; medial amygdala; hypothalamus

Introduction

In their classic paper Phoenix et al. (Phoenix et al., 1959) reported the results of 4 experiments that transformed our thinking about the mechanism controlling the development of sex differences in the capacity of rodent species (in this case, guinea pigs) to display sex-appropriate mating behaviors. Experiments 1 and 2 showed that administering testosterone propionate (TP) to pregnant guinea pigs reduced (defeminized) the capacity of their female offspring to show lordosis responses to manual (human) flank stimulation later in life following ovariectomy and injections of estradiol and progesterone. Phoenix et al. took the bold step (for 1959) of speculating in their Discussion that “An assumption seldom made explicit is that modification of behavior follows an alteration in the structure or function of the neural correlates of the behavior. We are assuming that testosterone or some metabolites act on these

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central nervous tissues in which patterns of sexual behavior are organized.” My assignment in this essay is to link the organizational changes in aspects of sexual behavior resulting from fetal exposure to TP to changes in the neural processing and actions of socially relevant olfactory cues (‘pheromones’). All of the lordosis responses studied by Phoenix et al. were triggered by manual flank stimulation from a human hand. Thus it seems unlikely that either the sex difference in lordosis responsiveness or the defeminizing actions of fetal TP on females’ lordosis behavior can be attributed to organizational actions of androgen on neural circuits that convey pheromonal inputs to parts of the brain that control lordosis behavior. Instead, the defeminizing effect of fetal androgen on subjects’ lordosis capacity more likely reflects a change in the neural responsiveness to ovarian steroids and/or in the processing of somatosensory inputs to the hypothalamus derived from manual flank stimulation.

Phoenix et al. also reported that fetal exposure to TP enhanced female guinea pigs’ capacity to display male-typical mounting behavior during tests given in adulthood. In Experiment 1 Phoenix et al. compared mounting behavior by gonadectomized subjects directed towards other male or female subjects during tests of lordosis behavior. When gonadectomized subjects were tested in the absence of adult hormone treatments only control males and females given TP fetally in doses/times that induced genital masculinization later displayed mounting; this behavior was not seen in control females or in females given TP fetally in doses/times that failed to masculinize the genitals. Interestingly, when these same groups of gonadectomized guinea pigs were treated in adulthood with estradiol and progesterone, control males and females as well as females given TP fetally displayed equivalent numbers of mounts directed towards the conspecifics used in lordosis testing. This latter observation is interesting in light of a recent proposal (Kimchi et al., 2007) that the circuitry controlling male-typical mating behavior lies dormant in the female rodent’s brain, with its function only being revealed when the female is deprived of pheromonal signaling following the disabling of vomeronasal organ (VNO) inputs to the olfactory nervous system (more on this below). In Experiment 3 Phoenix et al. reported that adult treatment of gonadectomized subjects with TP stimulated appreciable mounting behavior in control males and in females given fetal TP in doses/times that induced genital masculinization, with significantly less mounting being shown by control females. In Experiment 4 perinatal exposure to exogenous TP failed either to disrupt or enhance the capacity of male guinea pigs to display male-typical sexual behavior in adulthood (comparisons were made with untreated control males). Although details are missing from the methods section of Phoenix et al., I presume that the mounting behavior shown by subjects in their experiments was directed towards stimulus females that had been ovariectomized and primed with ovarian hormones so as to make them both attractive to conspecifics as well as receptive when mounted. There is now a substantial body of literature for rodent species that links male-typical attraction to estrous females as well as the male-typical capacity to display mounting and other male sex behaviors to male-typical anatomical and functional attributes of the main as well as accessory olfactory projections to the medial amygdala (Me) and the bed nucleus of the stria terminalis (BNST) as well as subnuclei of the hypothalamus, including the medial preoptic area (mPOA) and ventromedial hypothalamic nucleus (VMN). Phoenix et al. hinted at this forthcoming body of neuroscience research (to be reviewed, below) when they stated that “...the existence of a bisexuality is assumed. We suggest, however, that in the adult this bisexuality is unequal in the neural tissues as it is in the case of the genital tissues.”

The two olfactory nervous systems in mammals

Until recently, it was widely believed that pheromones that influence sexual attraction as well as mating behavior are exclusively detected in terrestrial vertebrates by the VNO, which is housed in a cartilaginous sheath in the roof of the mouth (Fig. 1). Pheromonal odorants, dissolved in nasal mucus, are actively pumped into the lumen of the VNO (Meredith and O’Connell, 1979) where they stimulate electrical activity in sensory neurons which express

two different families of G-protein linked receptor genes (Herrada and Dulac, 1997; Matsunami and Buck, 1997). In addition, VNO sensory neurons express transient receptor potential C2 (Trpc2) channels which generate action potentials after odorant stimuli bind to receptor proteins (Liman et al., 1999). VNO sensory neurons in mice are activated by urinary odors (Halem et al., 2001; Leinders-Zufall et al., 2000) as well as secretions of the extra-orbital lacrimal glands (Kimoto et al., 2005) from either same- or opposite-sex conspecifics. Different studies have specified the pheromonal ligands that activate VNO neurons as being major urinary proteins (MUPS) (Chamero et al., 2007), lacrimal gland secretions (Kimoto et al., 2005), as well as small, non-volatile peptide ligands for the major histocompatibility complex (MHC) class I molecules (Leinders-Zufall et al., 2004). VNO sensory neurons project axons to glomeruli located in the ipsilateral accessory olfactory bulb (AOB). These glomeruli are, in turn, innervated by dendrites from AOB mitral cells that extend axons to the 'vomeronasal amygdala' (Keverter and Winans, 1981a), which includes the Me (anterior and posterodorsal subdivisions) and the posteromedial cortical nucleus. Luo et al. (Luo et al., 2003) used *in vivo* recordings to show that AOB mitral cells were preferentially activated in mice of both sexes only when subjects made direct nasal contact with either the anogenital or facial regions of conspecifics. Urinary volatiles and other non-pheromonal volatile environmental odorants (e.g. food odors) failed to stimulate AOB mitral cell activity. Thus VNO receptor neurons respond preferentially to relatively heavy, non-volatile pheromones (although small MHC peptide ligands may be an exception to this rule). Information about pheromonal cues is further conveyed via a vomeronasal projection pathway extending from the Me to the BNST, mPOA, and VMH as well as the ventral tegmental area (VTA), the location of dopamine neurons that project to the nucleus accumbens (NAc) core and shell regions. A wealth of evidence suggests that many of both the behavioral and neuroendocrine effects of non-volatile pheromones are mediated by this VNO projection pathway. Neurons in different segments of this pathway express the transcription factor, Lhx6, and appear selectively to convey pheromonal inputs relevant to both behavioral and neuroendocrine aspects of reproduction (Choi et al., 2005).

Several lines of research (details below) suggest that volatile pheromones detected by olfactory receptor neurons (ORNs) in the main olfactory epithelium (MOE) also gain access to the above-mentioned VNO projection pathway at the level of the Me. Perhaps the earliest hint of a role for the main olfactory system in pheromone detection came from studies showing that zinc sulfate lesions of the MOE, but not VNO lesions, reduced the attraction of male hamsters to females' vaginal discharge (O'Connell and Meredith, 1984) and disrupted the preference of sexually satiated male hamsters for a novel estrous female (Johnston and Rasmussen, 1984). These results imply that males' detection of the relevant volatile pheromones must be initially detected by ORNs as opposed to VNO receptor neurons. ORNs extend axons to glomeruli located in the main olfactory bulb (MOB) (Fig. 1). These ORNs express up to 1000 different olfactory receptor genes in mice (fewer in humans) (Buck and Axel, 1991) that respond to a variety of combinations of volatile environmental odorants as well as MHC peptide ligands (Spehr et al., 2006). Future research will likely specify which particular receptor types detect particular pheromones emitted from urine or other body fluids. Recently, an additional family of trace amine-associated receptors (TAARs) was found to be expressed by murine ORNs (Liberles and Buck, 2006). These TAARs respond selectively to small amounts of volatile amines emitted from mouse urine as well as other body odorants. Volatile environmental odorants are detected by ORNs in the MOE whereupon they are processed via an input pathway that targets the anterior olfactory nucleus and olfactory cortices. In addition, there is long-standing evidence (Keverter and Winans, 1981b; Scalia and Winans, 1975) that MOB mitral cells project to the 'olfactory' amygdala which is comprised of the anterior cortical amygdalar nucleus (ACN) and the posterolateral cortical amygdalar nucleus. It seems likely that pheromonal inputs from the main olfactory system gain access to the olfactory amygdala whereupon intra-amygdalar connections further convey inputs to the 'vomeronasal' amygdala, which includes subnuclei of the Me. In addition, recent anatomical studies using female mice

(Kang et al., 2009) and rats (Pro-Sistiaga et al., 2007) identified a subset of mitral cells that abut ventral MOB glomeruli and extend axons directly to the Me. Volatile pheromones emitted from male, but not from estrous female, mouse urine stimulated Fos gene expression in a subset of these Me-projecting MOB mitral cells of female mice (Kang et al., 2009)—implying that a hard-wired circuit exists that conveys pheromonal information about gender from OSNs directly to the vomeronasal amygdala and on to the hypothalamus. On balance, the current consensus view of the anatomy of the olfactory nervous system holds that both the main and accessory systems detect and process pheromonal inputs that regulate heterosexual mate recognition, courtship, and sexual arousal in both sexes (Brennan and Zufall, 2006; Shepherd, 2006).

Display of male-typical mating behavior in both sexes

A critical result of Phoenix et al. (Phoenix et al., 1959) was that male guinea pigs, like females exposed to testosterone during fetal life, later showed significantly higher levels of mounting behavior than control females after all subjects had been gonadectomized and treated chronically with TP in adulthood. It must be noted; however, that under these circumstances even control females showed a low level of mounting behavior in tests with other receptive females. These behavioral results are consistent with the authors' conclusion that "... bisexuality is unequal in the neural tissues as it is in the case of the genital tissues." Stated differently, males and fetally androgenized females showed male-typical mating behavior more readily than females in response to the suite of adult endocrine and sensory (including presumably, olfactory) signals that impinged on the neural circuits controlling this behavior. Under the adult endocrine conditions that normally prevail, males of numerous mammalian species more consistently prefer to approach and mount an estrous female than do female subjects. The results of Phoenix et al. showed, however, that following adult treatment with estradiol followed by progesterone, gonadectomized control males, control females, and females treated fetally with TP all displayed equivalent (albeit relatively low) levels of mounting behavior. This outcome implies that normal females develop some capacity to display male-typical mating responses, although males display higher levels of this behavior than females when testosterone serves as the 'activational' (adult) sex hormone controlling expression of the behavior. Further evidence that the neural circuits controlling male-typical mounting behavior are present in female rodents comes from several reports that adult administration of high doses of TP to ovariectomized female rats (Baum et al., 1974; Beach, 1942; Emery and Sachs, 1975) and female mice (Dominguez-Salazar et al., 2004; Wersinger et al., 1997) stimulated high levels of mounting behavior. In female rats this mounting was often accompanied by intromission-like behavior even though females lack a penis. Thus females, like males, showed mounts with pelvic thrusting followed by a rapid backward lunge which in the male typically signifies a penile intromission into the stimulus female's vagina. Additional evidence that the female rat nervous system normally undergoes a degree of male-typical sexual differentiation derives from the observation (Bressler and Baum, 1996) that odors from soiled estrous female bedding stimulated similar levels of Fos expression in amygdalar and hypothalamic segments of the VNO projection pathway in gonadectomized, testosterone-treated adult male and female rats.

One interpretation of these various studies is that all female rodents inherently possess the underlying neural circuits controlling male sex behavior and that perinatal androgen exposure in males somehow enhances the ability of endocrine and olfactory stimuli to activate these circuits. An alternative explanation for the ability of female rodents to display male-typical mating behaviors is that female rats and mice (and perhaps females of other rodent species as well) normally receive appreciable fetal exposure to androgens, derived either from the placenta and/or from adjacent male fetuses. As a result, female rats and mice all may normally undergo a degree of male-typical brain and behavioral sexual differentiation. Empirical data

supporting this view derives from studies (Baum et al., 1991; Weisz and Ward, 1980) showing that plasma or whole body testosterone concentrations were equivalent in male and female rat fetuses across embryonic days 16–22 (22-day gestation) except for embryonic days 18–19, when testosterone levels were significantly higher in male fetuses. This profile of fetal testosterone levels differs dramatically from those observed in the ferret (Krohmer and Baum, 1989) in which male values were invariably significantly higher than those of females across the entire last quarter of a 41-day gestation. Interestingly, following gonadectomy and adult treatment with a high dose of testosterone, female ferrets consistently displayed significantly less neck grip, mounting, and pelvic thrusting behavior than male conspecifics (Baum, 1976; Baum et al., 1982). By contrast, fetal combined with early neonatal treatment of female ferrets with testosterone significantly enhanced their ability to display these male-typical mating behaviors up to the level displayed by control males (Baum et al., 1990). Taken together, these data imply that the inability of normal female ferrets to display appreciable male-typical mating behavior in response to adult treatment with testosterone reflects the absence of appreciable testosterone exposure, coupled to an absence of neural masculinization, during normal feminine fetal development. This contrasts with the rat (and perhaps with other rodent species including mouse and guinea pig) in which female fetuses are normally exposed to appreciable testosterone, likely of placental origin (Baum et al., 1991), so that in adulthood females display appreciable mounting behavior towards other females after adult ovariectomy and treatment with testosterone (references already cited, above). Normal, ovary-intact females may display mounting towards sluggish male conspecifics around the time of estrus (Afonso and Pfau, 2006) and towards other females during all phases of the ovarian cycle (Fang and Clemens, 1999). This latter display of mounting behavior may help maintain individual females' social status within female groups whereas the former type of mounting may be a form of feminine proceptive (sexual) behavior.

Recently, Kimchi et al. (Kimchi et al., 2007) proposed that (a) female mice possess all of the neural circuitry needed to display male-typical mating behavior, and (b) the reason females normally do not show mounting behavior is because pheromones detected by the VNO inhibit the activation of this dormant, male-typical neural circuitry. These authors based this conclusion on their observation that female mice with a homozygous null mutation of the *Trpc2* cation channel (*Trpc2* KO females) showed mounting and pelvic thrusting behaviors towards either another estrous female or a castrated male conspecific that received male urine swabbed on the back. The level of mounting behavior shown by *Trpc2*KO females, which were studied while ovary-intact and at unspecified times of the estrous cycle, resembled the mounting shown by *Trpc2*KO males. Additional transgenic females that expressed tau-LacZ driven by the olfactory marker protein (OMP) promoter as well as heterozygous *Trpc2*KO male and female mice that underwent surgical VNO removal (VNOx) also showed high levels of mounting towards both types of stimulus conspecific. Although no data were presented, Kimchi et al. stated that *Trpc2*KO females and males showed equivalent (high) levels of mounting behavior towards estrous females and castrated, urine-swabbed males when these stimuli were presented simultaneously. No data on wild-type or heterozygous *Trpc2*KO control animals of either sex that were presented simultaneously with these stimuli were apparently either obtained or published. Nor apparently were mice of either sex studied that received sham VNO surgeries.

In previous studies using female mice (Keller et al., 2006c) and female ferrets (Woodley et al., 2004) surgical removal of the VNO failed to augment subjects' display of mounting behavior towards male conspecifics (subjects' reactions to other female conspecifics were not studied in those experiments). In another recent study (K.M. Martel and M.J. Baum, unpublished results) adult female Swiss Webster mice from which the VNOs were surgically removed showed no augmentation of mounting behavior directed towards either an estrous female or a castrated, urine-swabbed male when these two types of stimulus mice were presented simultaneously. This was true in tests conducted when VNOx females (and VNO intact

controls) were tested while ovary-intact as well as after ovariectomy--either without or with treatment with estradiol and progesterone. Subsequently, treatment with testosterone significantly stimulated mounting directed primarily towards the stimulus female in VNO intact control females, and to a lesser extent in VNOx females. Likewise, lesions of the AOB (which receives inputs from the VNO) failed to augment mounting behavior above the level shown by sham-operated control females in tests given when females were ovariectomized and tested first without and then while receiving testosterone. It is noteworthy that Kimchi et al. (Kimchi et al., 2007) used ovary-intact females of different *Trpc2* genotypes in their studies. Although no data were shown, the authors stated that the mounting shown by *Trpc2* KO females was not influenced by stage of the estrous cycle. They also reported that plasma levels of free testosterone were significantly higher in homozygous *Trpc2* KO than in heterozygous *Trpc2* KO females (no data for wild-type females were presented). Likewise, no data on plasma testosterone levels were provided for VNOx females. Kimchi et al. took pains to downplay the possible contribution of the increased plasma testosterone levels seen in homozygous *Trpc2* KO females to their increased male-typical mounting behavior, arguing that plasma testosterone levels in gonad-intact males were 100 fold higher than in either group of females studied. However, in the absence of any effort to clamp circulating levels of testosterone in different groups of females at the time of behavioral testing, it is impossible to rule out the possibility that a small, but significant elevation in plasma testosterone stimulated the mounting displayed by homozygous *Trpc2* KO females. Indeed, as already reviewed, the work of Rissman and co-workers (Dominguez-Salazar et al., 2004; Wersinger et al., 1997) as well as Martel and Baum (2008, unpublished results) suggest that exogenous testosterone treatment effectively augmented the ability of ovariectomized (VNO-intact) adult female mice to display mounting behavior. The fact that normal female mice can show high levels of mounting behavior directed towards estrous stimulus females suggests that the circuits mediating this type of behavior are, as suggested by Kimchi et al. and others before them, present in females just as they are present in males. As proposed above, the existence of these circuits may reflect the actions of fetal testosterone exposure normally sustained by female rodent species or it may reflect a sexually monomorphic aspect of neural development which leads to the organization of male-typical circuits in both sexes.

It seems very unlikely that the ability of adult testosterone treatment to augment mounting behavior in adult ovariectomized female mice results from an androgen-mediated disruption of VNO signaling, which is a possible interpretation of the results of Kimchi et al. For example, there was no consistent effect of testosterone treatment on the expression of VR2 type receptor genes in the basal zone of the VNO in gonadectomized male and female mice (Alekseyenko et al., 2006), and if anything, adult testosterone treatment augmented the ability of pheromones from estrous female mice to stimulate immediate-early gene expression in VNO receptor neurons of castrated males (Halem et al., 2001). Our recent failure (K.M. Martel and M.J. Baum, unpublished results) to observe enhanced mounting in Swiss-Webster female mice following VNOx (regardless of endocrine state at time of testing) appears to differ from the report by Kimchi et al. (Kimchi et al., 2007) who found that VNOx females resembled *Trpc2* KO females by showing high level of mounting behavior directed towards either an estrous stimulus female or a stimulus male. It should be noted that Kimchi et al. included no sham-operated (VNOi) control females in their study. Thus the high levels of mounting behavior observed in VNOx females (like homozygous *Trpc2* KO females) contrasted with the lower level of mounting showed in surgically unmanipulated wild-type and heterozygous *Trpc2* KO females. It seems unlikely that high levels of mounting would be induced in wild-type control females by a sham VNO surgery, although Kimchi et al. did not directly assess this possibility. The female mice that received VNO lesions in the Kimchi et al. study were either of a mixed genetic background (C57BL/6J X 129/Sv) or were of the inbred C57BL/6JX strain. By contrast, the female mice studied by Martel and Baum were of the outbred, Swiss-Webster strain. It is

conceivable that these strain differences account for the different behavioral outcomes in the two studies. Future research will need to resolve this issue.

Kimchi et al. reported that their VNO removal surgeries caused extensive bleeding leading to the formation of blood clots in the nasal sinuses of their mice. These blood clots were removed by 7 daily sessions of saline irrigation given post-surgically before the onset of behavioral testing. In our experiments (K.M. Martel and M.J. Baum, unpublished results; Keller et al., 2006c; Pankevich et al., 2004; Pankevich et al., 2006a; Pankevich et al., 2006b) as in studies by other workers (Bean, 1982; Beauchamp et al., 1982; Clancy et al., 1984; Wysocki et al., 1982), such nasal saline irrigation after VNOx was not carried out. This raises the question of whether the inability of VNOx surgery to augment mounting behavior in our female mice reflected a deficiency in the detection of pheromones by subjects' MOE due to the formation of blood clots? Indeed, this possibility was explicitly suggested by Kimchi et al. (Kimchi et al., 2007) when referring to the results of two (Keller et al., 2006c; Pankevich et al., 2004) of our previously published studies. We have always waited at least 2 weeks after VNOx surgery before conducting behavior tests. It seems unlikely that putative blood clots which occluded the MOE (if, indeed, they ever existed) were retained longer than 2 weeks post-operatively in any of our studies in so far as VNOx mice (either males or females) in all of these experiments successfully performed behavioral tests showing that they could successfully discriminate volatile urinary odors from male vs estrous female mice. The main olfactory system had to be functional in these VNOx subjects in order for them to perform these olfactory discriminations. In addition, Pankevich et al. (Pankevich et al., 2004) found that VNOx male mice displayed male sex behavior at high levels that resembled the behavior of VNOi controls. This implies that there was no deficit in MOE function in so far as disrupting MOE signaling is known to block the display of male sex behavior by male mice (Edwards and Burge, 1973; Keller et al., 2006b; Mandiyan et al., 2005; Yoon et al., 2005). These results together with our recent unpublished data (K.M. Martel and M.J. Baum) suggest that the inability of VNOx as well as AOB lesions to augment females' male-typical mounting behavior did not reflect an undetected, post-surgical occlusion of the MOE by blood clots. Clearly, the hypothesis that VNO inputs tonically inhibit the function of neural circuits controlling male sex behavior in female mice (Kimchi et al., 2007) needs further testing by additional independent groups of investigators before it can be accepted.

Role of sexually dimorphic VNO-accessory olfactory signaling in the control of male-typical courtship and mating behaviors

Early work of Gorski et al. (Gorski et al., 1978) established that the volume of a sub-nucleus of the rat mPOA is sexually dimorphic—with the nucleus being 3–4 times larger in males than in females. Subsequent work of McCarthy et al. (Amateau et al., 2004; Amateau and McCarthy, 2002; Amateau and McCarthy, 2004) showed that the proliferation of dendritic spines on mPOA neurons is greater in male than in female rats as a result of a signaling cascade that begins with estradiol, formed neonatally in the male hypothalamus via the local aromatization of testosterone. Estradiol stimulates prostaglandin-E₂ synthesis which, in turn, upregulates AMPA-kainate glutamate receptors leading to the male-typical proliferation of mPOA dendritic spines. Guillamon et al. (Guillamon and Segovia, 1997) reported that sex dimorphisms also exist in the volume (male larger than female) of several brain regions that convey VNO inputs to the rat mPOA. These regions included the VNO neuroepithelium itself as well as the AOB, subnuclei of the medial amygdala (also see Cooke et al., 1999), and the BNST. These anatomical results set the stage for a series of functional neuroanatomical experiments that monitored the expression of the immediate-early genes (IEGs), c-fos and EGR-1, in different segments of the VNO projection pathway to the hypothalamus in male and female subjects following exposure to pheromonal cues from same- vs opposite-sex conspecifics. Even at the level of the VNO sensory neurons, a sexually dimorphic IEG response

to pheromonal cues was detected in mice (Halem et al., 2001; Halem et al., 1999). Another study (He et al., 2008) monitored calcium release from mouse VNO sensory neurons shown in response to male vs female urinary pheromones. No sex dimorphism in calcium release was observed in these neurons, although female urinary pheromones induced calcium release in VNO neurons of either sex more readily than male urinary pheromones.

Numerous studies using mice, rats, and ferrets have shown a sex dimorphism in Fos responses to male odors (emitted either from soiled bedding or from urine applied to the nose) in brain regions that receive VNO inputs, including the MePD, the BNST, mPOA, and VMH. The earliest such work was carried out in the rat (Bakker et al., 1996) and hamster (Fiber and Swann, 1996) and was subsequently extended to the ferret (Kelliher et al., 1998; Wersinger and Baum, 1997) and mouse (Halem et al., 1999; Wersinger and Rissman, 2000). In the rat the existence of a sex difference in the ability of male pheromones to stimulate Fos-expression in the VNO projection circuit to the mPOA was found to depend on the neonatal actions of estradiol, formed from circulating testosterone, in the male (Bakker et al., 1996). Thus neonatal treatment of male rats with the aromatase inhibiting drug, ATD, caused these subjects later in life to show a female-like profile of Fos expression in both the BNST and mPOA after exposure to soiled male bedding. By contrast, in mice it appears that perinatal androgen receptor activation accounts for the sex dimorphism in Fos expression seen in the VNO-projection targets after exposure to male pheromones. Thus androgen receptor –deficient male mice (tfm) showed a female-like profile of Fos expression in the MPN in response to pheromones emitted from soiled male bedding (Bodo and Rissman, 2007). In addition, neonatal treatment of female mice with the androgen receptor agonist, DHT, transformed subjects' hypothalamic Fos responses to male pheromones later in life so as to make them more like that of control males (Bodo and Rissman, 2008).

The above studies documented a sex dimorphism in neural responses to conspecific pheromones that correlated with a sex difference in the preference of males and females to approach and investigate odor cues of the 2 sexes that were deposited in the environment. Typically, males prefer to investigate female odors whereas estrous female prefer to investigate male odors. Several studies showed that VNOx of either female (Keller et al., 2006c) or male (Pankevich et al., 2004; Pankevich et al., 2006b) mice reduced subjects' preference to investigate opposite-sex vs same-sex pheromonal cues presented in either the home cage or in Y-maze tests. Likewise, in female hamsters (Petrulis et al., 1999) and male guinea pigs (Beauchamp et al., 1982) VNOx reduced the preference to investigate opposite-sex odors. We (Pankevich et al., 2004; Pankevich et al., 2006b) have argued that these results imply that VNO signaling plays a critical role in enhancing subjects' motivation to remain in close proximity to pheromonal cues deposited in the environment by the scent marking activities of opposite-sex conspecifics. This conclusion contrasts with that of Dulac and co-workers who have argued that VNO signaling in mice is mainly responsible for the ability of both males and females to discriminate the sex of conspecifics. Thus Trp2c KO male mice (Stowers et al., 2002) failed to display aggression towards male intruders but instead attempted to mount them. Likewise, mutant males displayed ultrasonic vocalizations indiscriminately towards conspecifics of both sexes and also indiscriminately mounted an estrous female and a castrated male whose back was swabbed with male urine when these stimuli were presented simultaneously. A similar profile of behavioral results was independently obtained in Trpc2 KO males by another group of workers (Leypold et al., 2002). Subsequently, Kimchi et al. (Kimchi et al., 2007) reported that Trpc2 KO female mice (like Trpc2 KO males) displayed indiscriminate mounting behavior towards estrous female and male conspecifics, and suggested, again, that VNO signaling is required in order for mice to discriminate conspecifics' gender. None of these studies of Trpc2 KO male and female mice used behavioral methods to assess subjects' olfactory discrimination capacity or odor preferences. When this was done in male mice using home-cage habituation/dishabituation tests, it was readily apparent that VNOx males resembled VNOi controls in their

ability to show a dishabituation response when urinary odors from a female were presented following a sequence of presentations of male urinary odors to which subjects quickly habituated (Pankevich et al., 2004). Likewise, VNOx female mice retained their ability to discriminate between male and female odors (Keller et al., 2006c). These latter results resemble earlier reports (Beauchamp et al., 1982) that male guinea pigs retained the ability to discriminate male and female urinary odors and that female hamsters retained the ability to discriminate male and female body odors (Petrulis et al., 1999) after VNOx. More recently, we (Woodley et al., 2004) found that female ferrets retained their ability to discriminate male and female urinary as well as anal scent gland odorants following VNOx—although as in mice, the preference of VNOx female ferrets to persist in their investigation of male urinary odors was significantly reduced compared to VNOi controls. We conclude that the main olfactory system is primarily responsible for monitoring volatile pheromonal signals that communicate information about gender and endocrine state—allowing conspecifics reliably to discriminate sex in the absence of any VNO signaling.

Early studies showed that removal of the olfactory bulbs eliminated sexual behavior in male mice (Rowe and Edwards, 1972), rats (Heimer and Larsson, 1967), and hamsters (Murphy and Schneider, 1970), suggesting that olfactory inputs are essential for masculine sexual arousal and mating performance. A subsequent study (Powers and Winans, 1975) showed that VNO lesions partly disrupted male sex behavior in hamsters, although the most dramatic disruption of sexual behavior was seen after both main olfactory and VNO signaling were disrupted. Meredith (Meredith, 1986) later found that a significant attenuation of mating behavior occurred after VNOx of male hamsters, provided the surgery was carried out prior to (but not after) males had received mating experience. Meredith interpreted this result to mean that VNO inputs at the time of first mating condition the main olfactory system to respond later to olfactory inputs in the absence of a functional VNO. In this way, the main olfactory system assumes the role of the VNO in providing the pheromonal signaling required for male sexual arousal and resultant mating performance (more on this, below). A similar argument has more recently been made in studies using female mice (Martinez-Ricos et al., 2007; Martinez-Ricos et al., 2008; Moncho-Bogani et al., 2005) in which these authors argued that VNO—accessory olfactory system's processing of male odors is hard wired so that it results in sexually naïve females being attracted to non-volatile male urinary odors deposited in the environment. These authors suggested that as a result of this experience with VNO odor inputs (direct nasal contact with male odors in the absence of mating will suffice), the main olfactory system somehow learns about the significance of these male odors so that females subsequently express a preference for volatile male odors that are detected solely by the main olfactory system.

Early studies carried out using male mice (Clancy et al., 1984; Wysocki and Lepri, 1991) suggested that VNOx disrupted mating behavior. However, the results of these studies were variable, and were carried out under less than optimal circumstances (e.g., day time instead of night time testing; short 20–30 min. tests) for masculine mating performance. It is clear from the results of the two studies of Trpc2 KO male mice (Leypold et al., 2002; Stowers et al., 2002) that, if anything, mounting performance was enhanced by genetic disabling of VNO neuronal signaling. In a similar vein, Pankevich et al. (Pankevich et al., 2004) found that VNOx of sexually naïve male mice had absolutely no disruptive effect on their mating performance in tests with estrous females. These tests were conducted during the dark phase of the light/dark cycle and involved a prolonged (4h) interaction between the VNOx male subjects and a stimulus female in estrus. Taken together, these results suggest that VNO signaling is not required for sexual arousal and complete mating behavior in male mice. Instead, the available evidence suggests that VNO signaling motivates males to remain in the proximity of female pheromonal cues—an action that likely contributes to eventual mating success.

Role of sexually dimorphic main olfactory system signaling in the control of male-typical courtship and mating behaviors

In contrast to the case of VNO sensory neurons, there is no evidence that sensory neurons in the MOE are sexually dimorphic in any manner. These OSNs project to glomeruli in the MOB which, in turn, are innervated by abutting mitral cells that extend axons mainly to the olfactory cortex. In addition, it has been known for some time (Davis et al., 1978; Scalia and Winans, 1975) that a subset of MOB mitral cells project to targets in the posterolateral cortical nucleus (PLCN) and anterior cortical nucleus (ACN), which are subnuclei of the 'olfactory' amygdala (Kevetter and Winans, 1981b). Intra-amygdalar connections convey inputs, in turn, from these regions to nuclei of the 'vomeronasal amygdala', including the Me and the postero-medial cortical amygdala (PMCN) where electrophysiological evidence of a convergence of VNO and MOE inputs has been reported (Licht and Meredith, 1987). Recently, however, studies in rat (Pro-Sistiaga et al., 2007) and mouse (Kang et al., 2009) have identified a subset of MOB mitral cells that send axons directly to the Me. In mice these MOB mitral cells are located in the ventral and medial aspects of the MOB where they abut glomeruli that are activated (express Fos in periglomerular cells) in response to volatile urinary odors from either male or female conspecifics (Martel and Baum, 2007; Schaefer et al., 2001). Interestingly, however, the subset of MOB mitral cells that project to the Me in female mice expressed Fos only after exposure to urinary volatiles from male conspecifics as opposed to urinary volatiles from other females or from a predator, the cat (Kang et al., 2009). More research is needed to determine whether in male mice there is a similar selectivity of MOB mitral cells responses to opposite-sex urinary odors. Our results from female mice raise the possibility that there is a mechanism at work in the MOB which makes the small number of mitral cells that project directly to the Me selectively responsive to reproductively salient, opposite-sex urinary pheromones.

What is the possible functional significance of the detection and processing of opposite-sex urinary volatiles by the main olfactory system? One consequence may be indirectly to stimulate the functional activity of the accessory olfactory system, including the AOB and its inputs to the vomeronasal amygdala and on to the hypothalamus. Evidence supporting this view comes from two recent reports: In an initial study (Martel and Baum, 2007) gonadectomized male and female mice (given no hormone replacement treatments) that were exposed to opposite-sex urinary volatiles showed a significant increase in Fos expression in the AOB mitral and granule cell layers. No such response was seen in mice exposed to same-sex urinary volatiles. Interestingly, in both sexes the AOB Fos response seen after exposure to opposite-sex odors was absent in subjects in which the MOE had been previously damaged by intranasal infusion of zinc sulfate. It seems unlikely that the AOB responses induced in MOE-intact mice by urinary odors reflected a detection and activation of VNO sensory neurons by these volatile pheromonal cues. First, the best evidence available suggests that these volatile odorants probably do not reach and/or activate VNO neurons in the absence of subjects' direct nasal contact with the chemical stimulus (Luo et al., 2003). Second, (Martel and Baum, 2007) direct nasal application of male urine stimulated significant AOB Fos expression in female subjects previously given zinc sulfate lesions of the MOE, suggesting that intranasal application of this toxin probably did not inadvertently damage VNO sensory neurons. This implies that the absence of an AOB Fos response to urinary volatiles in females previously given intra nasal zinc sulfate to destroy the MOE cannot be explained by unwanted damage to the VNO sensory neurons that project to the AOB. Additional preliminary data (K.M. Martel and M.J. Baum, unpublished results) showed that equivalent levels of Fos expression occurred in both the mitral and granular AOB cell layers in VNO-lesioned and VNO-intact female subjects that were exposed to male urinary volatiles.

A recent anatomical study showed that a small subset of MOB mitral cells project to glomeruli located in the rat AOB (Larriva-Sahd, 2008); however, there is no functional evidence that

MOE inputs are conveyed directly from the MOB to the AOB in either rat or mouse. This fact raises the question of how pheromonal cues detected by the MOE are able to stimulate Fos expression in the AOB? Information about opposite-sex pheromones may be conveyed to the AOB via centrifugal inputs that originate someplace in the MOB olfactory projection pathway. We (Martel and Baum, 2009) recently confirmed a previous report (Barber, 1982) that centrifugal inputs to the mouse AOB originate in several amygdalar nuclei including the anterior Me (MeA) and the bed nucleus of the accessory olfactory tract. Interestingly, only neurons in the female MeA, which were labeled by previous intra-AOB injections of the retrograde tracer, cholera toxin B (CTB), expressed Fos following exposure to male urinary volatiles. No such stimulation of Fos expression in AOB-projecting MeA neurons was seen in female mice exposed to same-sex urinary volatiles or to volatile cat urinary or food odors. Thus both in MOB mitral cells that project to the MeA as well as in a subset of MeA neurons that project to the AOB, Fos expression was selectively stimulated in response only to opposite-sex urinary volatiles. These results suggest that reproductively salient pheromonal cues, detected in a sexually differentiated manner by the main olfactory system, gain access to the AOB and downstream forebrain targets in the VNO projection pathway to the hypothalamus. While more research is needed to assess the possible significance of this type of pheromonal communication, one study (Jakupovic et al., 2008) showed that bilateral electrolytic lesions in the AOB significantly reduced the interest of male mice in investigating urinary volatiles from estrous female as opposed to gonadally intact male conspecifics. The processing of opposite-sex volatile pheromonal cues, which are detected by the main olfactory system and subsequently processed via the AOB, may contribute to sexual attraction and eventual reproductive success in mice.

Several lines of evidence point to a sex dimorphism in the detection by the main olfactory system as well as the central processing of pheromonal cues that likely play a central role in mate recognition in several mammalian species. The earliest indication of such an effect was seen in studies that examined the ability of male and female pigs to respond to the putative male pheromone, androstenone, which both attracts estrous sows to males and facilitates their receptive responsiveness to mounts received from a male (Signoret, 1970). Dorries et al. (Dorries et al., 1995) used an operant, food-motivated task to show that sows' detection threshold for androstenone was 5 times lower than that of boars, with the threshold of neonatally castrated males resting between these two extremes. It seems likely that this observed sex difference in androstenone detection thresholds reflects a difference in main, as opposed to accessory olfactory system function because occlusion of the VNO duct failed to modify sows' ability to perform an operant task in which androstenone served as the discriminative stimulus (Dorries et al., 1997). There was no group difference in the ability to detect decreasing concentrations of a floral odor, geraniol. One limitation of the work of Dorries et al. is that they used gonadally intact, adult male and female pigs. Thus it is impossible to know whether the greater sensitivity of sows to androstenone reflects a difference in circulating sex steroids at the time of testing or a hard-wired sex dimorphism in the function of the olfactory nervous system that resulted from the perinatal and/or prepubertal ('organizational') actions of sex hormones in the male, such as those responsible for the sexual differentiation of mating capacity (Phoenix et al., 1959).

Recent work using mice has circumvented this latter problem and provided evidence that there is a functional sex difference in the ability to detect and process urinary volatiles that may be hard wired as a result of perinatal brain sexual differentiation. Thus Sorwell et al. (Sorwell et al., 2008) compared the ability of gonadectomized adult male and female mice to use progressively lower concentrations of male mouse urinary odors as a discriminative stimulus for obtaining a food reward. When no sex hormones were given at the time of testing, gonadectomized males and females showed similar detection thresholds for male urinary volatiles. However, after 1 week of estradiol treatment to all subjects, ovariectomized females

successfully detected significantly lower concentrations of male urine than did castrated males. Finally, when gonadectomized subjects were retested 2 weeks after cessation of estradiol injections, there was, again, no sex difference in subjects' ability to detect male urinary odors. These results in mice (Sorwell et al., 2008) and the previous results from pigs (Dorries et al., 1995) imply that perinatal events (e.g., actions of testosterone and/or estradiol in the developing male nervous system) normally defeminize males' capacity to respond to adult estradiol treatment which in females normally enhances their ability to detect male pheromones. At present, there no information about where in the circuitry of the main olfactory system this type of sexual differentiation of pheromonal detection capacity takes place. One possibility involves the nor-adrenergic, centrifugal input to the granule cell layer of the MOB which originates in the locus coeruleus (LC) (Shang and Dluzen, 2001). The number of nor-adrenergic neurons in the LC of both rat (Guillamon et al., 1988) and mouse (Pendergast et al., 2008) is greater in females than in males, and there is a suggestion that LC inputs to the MOB modify the plasticity of olfactory responses to urinary odors (Shea et al., 2008).

Some of the earliest evidence for a role of the main olfactory system in the control of sexual behavior came from experiments conducted in male and female mice (Edwards and Burge, 1973) in which zinc sulfate lesions of the MOE disrupted mating in both sexes. This effect of zinc sulfate has recently been replicated and extended in mice of both sexes (Keller et al., 2006a; Keller et al., 2006b). Genetic disruption of signaling pathways of OSNs in male mice (Mandiyan et al., 2005) also disrupted mating. By contrast, peripheral anosmia in several other species, including male hamster (Powers and Winans, 1973), male dog (Hart and Haugen, 1971), male rhesus monkey (Goldfoot et al., 1978), and male and female ferret (Kelliher and Baum, 2001) failed to disrupt mating behavior when conspecifics were placed together in a small chamber. Male and female mice normally prefer to approach and investigate urinary pheromones from opposite-sex conspecifics, and this preference was totally eliminated, again in both sexes, after zinc sulfate lesions of the MOE (Keller et al., 2006a; Keller et al., 2006b). These behavioral observations correlate with the finding (Martel and Baum, 2007) that urinary volatiles from male and female mice activated different clusters of glomeruli located primarily in the ventral portion of the MOB in both male and female subjects. Thus at the level of the MOB, mice of both sexes encode a distinction between urinary pheromones from the two sexes. Presumably, the main olfactory system of mice of both sexes responds differently to pheromones from males and females because the composition of these candidate pheromonal molecules differs significantly (Jemiolo et al., 1991; Liberles and Buck, 2006; Lin et al., 2005). In the ferret exposure to volatile anal scent gland odorants from breeding male vs estrous female conspecifics also stimulated different profiles of glomerular activation in the ventral MOB of both sexes (Woodley and Baum, 2004). Presumably the MOB glomeruli of ferrets respond differently to anal scent gland odorants from the 2 sexes because their composition differs chemically in males and females (Zhang et al., 2005). Again in ferret as in mouse, induction of peripheral anosmia, in this instance by physically occluding the MOE with dental impression material (ferrets, unlike mice, are able to breathe readily via the mouth following nares occlusion), blocked the preference of both male and female subjects to approach and interact with opposite-sex conspecifics in Y-maze tests of partner preference, even when subjects were given an opportunity to have a brief physical interaction with stimulus males and females after each operant trial (Kelliher and Baum, 2001). Clearly, this effect of peripheral anosmia on mate recognition reflected a selective disruption of MOE, and not VNO, function in that VNO lesions in female ferrets failed to duplicate the disruptive effects of nares occlusion on subjects' preference to seek out opposite-sex (male) odors in Y-maze tests (Woodley et al., 2004). We (Woodley and Baum, 2003) obtained no evidence that male and female ferrets showed differential sensitivity to low concentrations of anal scent gland odorants in home cage habituation/dishabituation tests. This implies that the initial detection and processing of pheromonal cues (derived from anal scent glands and/or urine) in the ferret is similar in the 2

sexes, and that the differential preference of males and females to approach these odors reflects some difference in their central processing.

Two early studies (Kelliher et al., 1998; Wersinger and Baum, 1997) showed that the central processing of odors emitted from male soiled bedding, which are detected via the main olfactory system, is sexually differentiated at the level of the ferret's mPOA and VMH. Thus, male odors stimulated Fos expression in the MOB, but not the AOB, of both sexes. Likewise, similar Fos responses to these odors were seen in the Me and BNST of the 2 sexes whereas only females showed significant Fos responses to male odors in the mPOA and VMH. These latter sex differences in pheromone-induced Fos responses in the hypothalamus resemble the sex differences described earlier in the responses of rat, hamster, and mouse to male odors that were presumably detected by the VNO-AOB whereas in ferrets the primary detection/processing system seems to be the MOE-MOB. Our observation (Kelliher et al., 1998) that female ferrets showed a significant Fos response to male, but not to other female, body odors in the VMH raised the possibility that the processing of male pheromones by VMH neurons contributes to female-typical preference to approach male as opposed to female body odors (Chang, 2000). More recent results (Robarts and Baum, 2007) support this view: Electrolytic lesions placed in the VMH, but not at the border of the medial preoptic area and anterior hypothalamus (POA/AH), disrupted the normal preference of female ferrets to approach male as opposed to female body odors in Y-maze tests. These VMH lesions had no impact on females' ability to discriminate male and female anal scents or urinary odors in habituation/dishabituation tests, implying that their effect on females' preference to seek out male vs female odors reflected a motivational effect as opposed to an effect on females' olfactory discrimination capacity.

It is noteworthy that placing lesions in the POA/AH of female ferrets failed to affect their preference to seek out odors from a male (Robarts and Baum, 2007). In male ferrets the POA/AH contains a cluster of large neurons in nissl-stained brain sections that is not seen in females (Tobet et al., 1986b). This male nucleus (MN; so-named because it is seen only in male ferrets) of the POA/AH develops over the last quarter of the 41-day gestation period under the influence of estradiol, formed in the developing male nervous system via the aromatization of testosterone that is secreted by the fetal testes (Cherry et al., 1990; Tobet et al., 1986a). Either excitotoxic (Paredes and Baum, 1995) or electrolytic (Kinson et al., 1996) lesions centered in the MN-POA/AH of male ferrets caused them to prefer to approach and interact sexually with another male, as opposed to a female, in T-maze tests of partner preference. In both our recent (Robarts and Baum, 2007) and early (Paredes and Baum, 1995) studies, lesions placed in the POA/AH of female ferrets failed to affect their female-typical preference to approach and interact with a male, as opposed to a female, conspecific. Taken together, these results imply that sexually dimorphic neurons in the male ferret's POA/AH tonically inhibit circuits that control female-typical partner preference. When these neurons were destroyed in males, their partner preference profile reverted to the female phenotype. Note that the effects of selective lesions of the MN-POA/AH were restricted to motivational aspects of sexual behavior (Cherry and Baum, 1990). Such lesions failed to reduce the ability of male ferrets to display any aspect of male-typical mating behavior. This contrasts with the effects of larger POA/AH lesions in male ferrets and in essentially every other male vertebrate studied to date, in which deficits in male sex behavior have been reported following POA/AH damage (Hull et al., 2002).

Another study (Alekseyenko et al., 2007) using ferrets has also successfully linked sexually dimorphic features of POA/AH morphology to a male-specific preference for opposite-sex (female) pheromones. Thus, electrolytic lesions centered bilaterally in the sexually dimorphic MN-POA/AH caused male ferrets to switch their pre-operative preference to approach female odors in Y-maze tests so that lesioned males preferred to approach odors of other males. Postoperatively, these lesioned male ferrets showed an odor preference profile that resembled

that of control females. Finally, after completion of the behavioral testing, males with lesions centered in the sexually dimorphic MN-POA/AH showed a female-like profile of Fos expression in the mPOA—one of the brain regions in which previous studies (Kelliher et al., 1998; Wersinger and Baum, 1997) found that male bedding odors significantly augmented Fos expression in normal female ferrets. This latter result implies that the peripheral processing of main and/or accessory pheromonal inputs may be similar in the two sexes whereas the motivational salience of these signals differs in males and females because of the presence of sexually dimorphic morphological features of the POA/AH. The notion that a sex difference in the hypothalamic processing of pheromonal inputs leads to a sex-appropriate preference to seek out potential mates owes a continuing debt to the seminal paper of Phoenix et al. (Phoenix et al., 1959).

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Fig. 1. Schematic drawing that shows connections of the accessory (left) and main (right) olfactory systems in the mouse. Light shading shows areas that receive direct input from the main olfactory bulb; dark shading shows areas that receive direct input from the accessory olfactory bulb. In the amygdala, light shaded areas indicate the olfactory amygdala and dark shading indicates the vomeronasal amygdala. The open arrow in the main olfactory pathway represents a recently identified direct connection to the medial amygdala. Abbreviations: VNO, vomeronasal organ; AOB, accessory olfactory bulb; MeA, anterior medial amygdala; MePD, posterodorsal medial amygdala; PMCo, posteromedial cortical amygdala; ACo, anterior cortical amygdala; PLCo, posterolateral cortical amygdala; BNST, bed nucleus of the stria

terminalis – lateral (L) and medial (M) subdivisions; mPOA, medial preoptic area; VMH, ventromedial hypothalamic nucleus; VTA, ventral tegmental area; Acb, nucleus accumbens—core (C) and shell (S); MOE, main olfactory epithelium; MOB, main olfactory bulb; AON, anterior olfactory nucleus; OT, nucleus of the olfactory tract; Pir, piriform cortex; Ent, entorhinal cortex. This diagram was kindly provided by Diana Pankevich, Ningdong Kang and James Cherry.