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Internal and External Control of Instinctual Social Behaviors

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Internal and External Control of Instinctual Social Behaviors

by

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DISSERTATION

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Abstract

In sexually reproducing animals, innate sexually dimorphic behaviors are regulated internally by gonadal steroid hormones and other cues and by sensory cues from the external world, such as pheromones.

In mice, pheromones can be sensed by either the main olfactory epithelium (MOE) or the vomeronasal organ (VNO). The relative contribution of these two chemosensory subsystems to sexually dimorphic behaviors is not adequately understood. Using mouse strains genetically engineered to lack odorant-evoked signaling in either the MOE or VNO, we investigated the interaction between these systems in male mating behavior and in several female-typical behaviors. We found that the VNO inhibits aberrant male-typical mounting behavior in both males and females.

Pheromonal control of female-typical behaviors is complex, with a different requirement for MOE and VNO input for each behavior studied. While female sexual behavior is redundantly regulated by the MOE and VNO, maternal aggression requires both sensory epithelia to be functional. Maternal care of pups requires MOE function and is redundantly controlled by VNO signaling.

While olfactory input is necessary for initiating normal male mating behavior, subsequent steps are highly stereotyped and follow a genetically controlled pattern. Preliminary analysis of wild-type male mating demonstrates that the latency to begin mating predicts the likelihood of successful completion. Taken together with the data on chemosensory control of male mating, this highlights the stereotyped nature of the mating pattern

between the initiation of mounting and ejaculation as well as the importance of the first mount as the output of the choice to mate.

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Chapter One: Introduction

Sexually dimorphic behaviors

All animals exhibit innate social behaviors that are often used to ensure reproductive success. The innate nature of these behaviors makes them useful models for how neural circuits control behaviors. They can be observed in naïve animals with no prior social experience, implying that they are developmentally hardwired. However, display of such innate behaviors is tightly regulated by the internal physical milieu as well as by appropriate sensory cues from the external world. This tight regulation ensures that behaviors such as mating and aggression are displayed at appropriate times and circumstances. In mice, males and females show many differences in behaviors, including mating, territorial behavior, and parenting.

Mating

Male mating behavior in the mouse begins with olfactory investigation, particularly of the anogenital region. The male then begins mounting the female from behind, thrusting rapidly. Upon penetration (intromission), the thrusting pattern slows and becomes deeper. The mouse requires several bouts of intromission before ejaculating (Hull and Dominguez, 2007). This mating behavior is robustly elicited from naïve males upon introduction of a female in estrus.

Males also "sing", i.e. emit ultrasonic vocalizations to females or female odors but not to males or male odors (Nyby et al., 1977; Whitney et al., 1973). These vocalizations have

been compared in structure and complexity to birdsong (Holy and Guo, 2005). Females will choose to spend more time with a male capable of singing, suggesting that this is a courtship behavior that facilitates mating by keeping the female nearby (Pomerantz et al., 1983).

Female mating behavior consists of receptivity to a male's mounts. When receptive, a female responds to being mounted by a male by holding still and assuming a lordosis posture, with back arched and hindquarters and tail raised. This posture allows the male to intromit. In contrast, an unreceptive female will flee, kick, or rear and push the male away. The female may also remain still but disallow intromission by sitting down (McGill, 1962).

Female mice will mount other females in a manner similar to males, but they do so at a lower frequency than males (Wu et al., 2009). The purpose of this mounting behavior, if any, is not known.

Aggression

Male mice show territorial behavior, marking their territory by urinating in scattered spots throughout the space (Desjardins et al., 1973). Having claimed an area as their own, they will aggressively defend that territory against male conspecific intruders. Upon introduction of another male into his space, the resident sniffs the intruder and then begins attacking the intruder, chasing him and biting the back or flanks. This behavior is sometimes accompanied by tail rattling (Ginsburg and Allee, 1942; Miczek et al., 2001). Fighting among males living together eventually establishes a social hierarchy in which

dominant males are more successful at reproduction (D'Amato, 1988; Uhrich, 1938).

Female mice do not show this territorial behavior. Rather than scattering urine, they pool it in one or a few spots (Kimura and Hagiwara, 1985). Moreover, under most circumstances females will not show any aggression toward other mice. However, lactating females will protect their pups by attacking intruder mice. Unlike males defending a territory, they will attack either male or female intruders with equal probability (Gandelman, 1972). Males often attack and kill strange pups, which provides an evolutionary explanation for this maternal aggression (vom Saal and Howard, 1982).

Parenting

Like most mammals, mice show parental care toward their offspring. Mice build a nest and keep the pups inside it, which is important for the survival of pups too young to thermoregulate. While in the nest, they nurse and lick the pups. Pups separated from the nest emit ultrasonic distress calls, and mice showing parental behavior quickly return the pups to the nest.

Both male and female mice may show parental behavior; however, the control of these behaviors is sexually dimorphic, with the degree of sexual dimorphism being strain-dependent. In most laboratory strains, even most naïve females will show maternal behavior upon exposure to another female's pups (Lonstein and De Vries, 2000; Noirot, 1964). In contrast, many naïve males are infanticidal, attacking and biting pups to death, often while tail-rattling (Brooks and Schwarzkopf, 1983). When attacks are directed at 20-day-old pups, the behavior is qualitatively similar to inter-male aggression (vom Saal,

1985). Mating modulates this behavior, with infanticide initially rising in the days post-ejaculation but then plummeting to well below naïve rates at times when pups could conceivably have resulted from that mating. At 20 days after mating (approximating the length of mouse gestation), the majority of males have switched from infanticide to parental care and will build a nest and retrieve strange pups to it. Similarly, while rarely showing infanticide, females show a decrease in maternal behavior shortly after mating, followed by a return to pre-mating levels during gestation.

Hormonal control of sexually dimorphic behaviors

It has long been known that sexually dimorphic behaviors in mammals are dependent on gonadal steroid hormones, including testosterone, estrogen, and progesterone. Hormones exert their control both developmentally and in adulthood. In the male, testosterone is produced by the testes. It acts via the androgen receptor (AR). Testosterone can also be converted to estrogen by the enzyme aromatase, which is expressed in a sexually dimorphic pattern in the brain. In females, testosterone is present only at very low levels. Instead, estrogen and progesterone are produced in a cyclical pattern corresponding to the estrous cycle.

In males, a surge of testosterone around the time of birth is converted in the brain to estrogen, which then masculinizes the brain with respect to aggressive and territorial behaviors and de-feminizes mating behaviors. In adulthood, testosterone and estrogen act together to activate these male-typical behaviors. Females given estrogen at birth show male-like territorial marking and aggression with only wild-type ovarian hormones

but need testosterone in adulthood to reach male-like levels of these behaviors (Wu et al., 2009). Similarly, males lacking AR in the brain (and thus responding only to estrogen, not testosterone) show male-like behavior at lower levels than wild-type males (Juntti et al., 2010).

Like territorial behaviors, male-like mating behaviors are activated by both estrogen and testosterone. However, in contrast to those for aggression, the circuits for male mating behaviors appear to be intact in normal female mice. Exogenous testosterone given to wild-type adult females raises the rate of male-like mating to male levels (Edwards and Burge, 1971). Female mating behavior is dependent on the estrous cycle. During the estrous cycle, a rise in estrogen and progesterone acts on GnRH-releasing neurons in the hypothalamus, leading to a surge in luteinizing hormone (LH) from the pituitary, which in turn acts on the ovaries to trigger ovulation (Chappell and Levine, 2000; Mahesh and Muldoon, 1987). At the same time, estrogen and progesterone stimulate receptive behavior, which is thus synchronized to peak fertility (Mani et al., 1997).

The hormonal control of parental behavior in the mouse is still unclear. Postpartum female mice show more maternal behavior than do naïve virgins, suggesting that the hormonal fluctuations of pregnancy and birth may prime the female to show maternal behaviors immediately. In rats, estrogen and progesterone levels associated with late pregnancy increase maternal behaviors (Mayer et al., 1990). This may also be the case in wild mice, which – unlike inbred laboratory strains – are infanticidal when nulliparous. However, in laboratory strains, experience with pups is sufficient to bring virgin maternal behavior to postpartum levels, and this experience-based behavior change is estrogen-

independent (Stolzenberg and Rissman, 2011). Only nest-building behavior has been linked to estrogen and progesterone levels associated with pregnancy (Lisk, 1971). The sexual dimorphism in parental behavior shows some hormone dependence; male-typical infanticide appears driven in part by adult testosterone. However, in contrast to its effect on inter-male aggression, neonatal testosterone attenuates the effect of adult testosterone on infanticide (Gandelman and vom Saal, 1977; Lonstein and De Vries, 2000). Interestingly, the effect of adult testosterone on infanticide by females can be reversibly neutralized by the continued presence of pups (Gandelman and et al., 1973). Although progesterone is linked to increased or unchanged parental care in female rodents, progesterone and its receptor increase infanticide in males, implying a dimorphic response to progesterone that may be related to its receptor's dimorphic expression pattern in the brain (Schneider et al., 2003; Wagner et al., 2001; Yang, 2012).

Pheromones

Display of the sexually dimorphic behaviors described above depends not only on the internal state of the animal, but also on appropriate sensory cues from the external world. These behaviors are, for the most part, social behaviors. That is, they are interactions between more than one of the same species. Therefore, it is necessary for one animal to be able to identify the social and sexual status of conspecifics. For example, as described above, a male must distinguish between and adult female, an adult male, and a pup to determine whether to mate, fight, or parent.

In the mouse, an important source of information for social behaviors is chemosensory

cues from conspecifics, also known as pheromones. The term "pheromones" was defined by Karlson and Luscher (1959) as "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process" and was applied initially primarily to those emitted by insects. The original conception of a pheromone has not always corresponded well to vertebrate chemosensation, in which there is rarely, if ever, a tight linkage between a single sensory cue and a behavior (Kelliher, 2007). Though some have argued for a narrowing of the definition as it applies to vertebrates, we use a broad definition that includes any chemosensory cue emitted by members of a species that provide information about social and sexual status to other members of the same species. Notably, this definition does not distinguish between innate responses and learned cues or between single molecule signals and responses to mixtures.

Non-human vertebrate pheromones are often divided into priming and releaser pheromones. Releaser pheromones lead to a relatively quick and short-term change in behavior, while priming pheromones cause a relatively long-lasting physiological change. For example, in mice male odors, including certain major urinary proteins (MUPs), elicit almost immediate aggression from adult males toward the bearer of the odors, a releaser effect (Chamero et al., 2007). Odors from adult male mice also accelerate puberty in young females, a priming effect (Nishimura et al., 1979). Some would also consider as a separate category signaling pheromones, which provide information without necessarily releasing a specific behavior, though this cannot necessarily be cleanly separated from

releaser effects (Kelliher, 2007).

Main Olfactory Epithelium and Vomeronasal Organs

The rodent nose, like that of most vertebrates, possesses more than one chemosensory system, including the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). The MOE lines the posterior portion of the nasal cavity and responds to volatile odorants carried in the air the animal breathes. The vomeronasal organ is a tube at the base of the lateral septum. Molecules dissolved in mucus are actively pumped into the VNO for detection by the sensory neurons which line it (Meredith, 1994). This mechanism allows the VNO to access non-volatile molecules (via physical contact with an odor source) as well as volatile odorants. Traditionally, the VNO has been associated with detection of pheromonal cues while the MOE has been associated with other odorants, such as food. However, both have been shown to play a role in responding to pheromonal cues.

Main olfactory epithelium signaling

In the MOE, odorant molecules are sensed by olfactory receptors (ORs) in ciliated olfactory sensory neurons (OSNs) in the epithelium. These neurons are continually replaced, with a lifespan of weeks (Mackay-Sim and Kittel, 1991). Each OSN expresses a single receptor (or possibly a very few) monoallelically (Mombaerts, 2004). ORs are G protein coupled receptors (GPCRs). Binding of the odorant molecule to the receptor leads to activation of $G\alpha_{olf}$, which stimulates adenylyl cyclase 3 (AC3) to produce cAMP, which in turn opens cyclic nucleotide gated cation channels. The main, pore-forming

subunit of the channel is encoded by the X-linked gene *Cnga2* (Elsaesser and Paysan, 2005). This channel is necessary for detection of odorants by OSNs since mice with a targeted deletion in *Cnga2* show no response in OSNs to odor presentation (Brunet et al., 1996).

The mouse genome possesses around 1000 different olfactory receptor genes, most of which have no known ligand. A single odorant appears to often activate multiple receptors, while individual receptors may respond to multiple odorants (Saito et al., 2004). Olfactory receptor genes are divided into two classes whose expression is spatially distinct, with Class I receptors and a subset of Class II receptors expressed dorsally and the remaining Class II receptors expressed ventrally. Unlike the ventral portion of the MOE, the dorsal region has been linked to innate fear responses, and it is proposed that this subset of neurons may also mediate innate pheromonal responses (Kobayakawa et al., 2007).

OSN axons project to the main olfactory bulb (MOB) where those expressing the same receptor converge to one or a few glomeruli per bulb. The main olfactory bulb has connections to the taenia tecta (TT), the anterior olfactory nucleus (AON), the olfactory tubercle (OT), the pyriform cortex (PYR), the entorhinal cortex (EC), and the anterior cortical nucleus (ACN) and posterolateral cortical nucleus (PLCN) of the amygdala (Meredith, 1998).

Vomeronasal organ signaling

The vomeronasal organ (also known as Jacobson's organ) consists of a pair of tubes with

a crescent-shaped cross-section located on either side of the base of the nasal septum. In rodents, each tube communicates with the nasal cavity by a narrow duct. Odors are actively pumped into the organ where they can activate vomeronasal neurons (VNs). These neurons line the medial surface of the VNO lumen and possess microvillae where the receptors are located. VNs can be divided into two populations based on their position and expression of signaling molecules. VNs do not express the signaling machinery of the MOE (e.g. CNGA2). Those in the more apical layer of the epithelium express receptors from the V1R superfamily and $G\alpha_{i2}$, while those in the basal layer express receptors from the V2R superfamily and $G\alpha_o$ (Halpern, 1987; Halpern and Martinez-Marcos, 2003). Both populations express TRPC2, a member of the transient receptor potential family of ion channels. The VNOs of *Trpc2* knockout mice show extremely reduced response to odors (Leypold et al., 2002; Stowers et al., 2002).

The V1R (Dulac and Axel, 1995) and V2R (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997) families of receptors have no significant homology either with each other or with the ORs, though all three families are members of the GPCR superfamily. There are 100-200 genes in each VR family in the mouse in addition to many pseudogenes (Rodriguez et al., 2002; Young and Trask, 2007; Zhang et al., 2004).

Rodent vomeronasal neurons project to the accessory olfactory bulb (AOB). As in the MOB, neurons converge to glomeruli, and the apical-basal division in the VNO is reflected in the anterior-posterior axis of the AOB. However, neurons expressing a single V1R project to multiple glomeruli. From the AOB, connections are made to the medial

amygdala (MeA and MeP), posteromedial cortical nucleus (PMCN), bed nucleus of the stria terminalis (BNST), and nucleus of the accessory olfactory tract (NAOT) (Meredith, 1998). Several of these regions are implicated in sexually dimorphic behaviors (Choi et al., 2005; Simerly, 2002).

In addition to these downstream brain regions, the VNO itself is capable of responding to conspecific odors in a sexually dimorphic manner implying that sexual dimorphism in behaviors begins at the level of sensory perception (Halem et al., 2001; Herrada and Dulac, 1997; Martel and Baum, 2007; Thompson et al., 2004).

Role of MOE and VNO in sexually dimorphic behaviors

Many studies have demonstrated the importance of olfaction via both the MOE and the VNO in sexually dimorphic behaviors. Early studies found that olfactory bulbectomy, which disrupts signaling from both the MOE and the VNO, abolished or diminished male and female mating behaviors, inter-male aggression, and maternal care in mice (Gandelman et al., 1971; Ropartz, 1968; Rowe and Edwards, 1971, 1972; Thompson and Edwards, 1972; Zarrow et al., 1971). Since then, efforts have been made to clarify the roles of each system in pheromonal control of these behaviors.

Mating

Male mating is severely reduced by genetically eliminating MOE signaling via the mutation of either *Cnga2* or *AC3* (Mandiyan et al., 2005; Wang et al., 2006). Chemically ablating the MOE with the herbicide dichlobenil phenocopies the genetic ablations (Yoon

et al., 2005). (This contrasts with earlier results which found that chemical ablation of the MOE with $ZnSO_4$ had no effect on male mating (Edwards and Burge, 1973)).

Surgical disruption of the VNO was initially reported to decrease male mating behavior and ultrasonic vocalizations toward females (Clancy et al., 1984; Wysocki et al., 1982). Yet abolishing VNO function by knocking out *Trpc2*, contrary to expectations, not only did not diminish male mating, but also eliminated sex discrimination in males such that they attempted to mate with males as well as females (Leypold et al., 2002; Stowers et al., 2002).

Female receptivity, on the other hand, appears thus far to require both MOE and VNO input. Surgical disruption of the VNO or $ZnSO_4$-mediated disruption of the MOE each diminish but do not eliminate lordosis behavior in female mice, with the effects of $ZnSO_4$ intermediate between no treatment and complete bulbectomy (Edwards and Burge, 1973; Keller et al., 2006a; Keller et al., 2006b).

In contrast to effects on receptivity, only the MOE is required for females to continue to prefer to investigate both volatile and non-volatile male odors over female odors (Edwards and Burge, 1973; Keller et al., 2006a). In the absence of the VNO, the MOE is sufficient for a preference for male volatile odors (Keller et al., 2006b). Interestingly, however, there is evidence that forming the preference for male volatile odors is dependent on previous experience of non-volatile odors (Ramm et al., 2008), suggesting that innate recognition of pheromones by the VNO may trigger the animal to learn associated MOE-dependent cues that then act as pheromones.

Aggression

Both the MOE and the VNO appear to be necessary for inter-male aggression. $ZnSO_4$-treated, *Cnga2*$^{-/Y}$, and *AC3*$^{-/-}$ male mice show diminished aggression toward other males, as do those mutant for *Trpc2* or with the VNO surgically disrupted (Bean, 1982; Edwards et al., 1972; Leypold et al., 2002; Mandiyan et al., 2005; Maruniak et al., 1986; Stowers et al., 2002). Male mice with surgical lesions of the VNO have also been found to have decreased urine marking, though previous mating experience reduces or perhaps eliminates this difference (Clancy et al., 1984; Wysocki and Lepri, 1991).

Maternal aggression also seems to require both the MOE and the VNO, since mothers with the VNO removed or who are mutant for either *Trpc2* or *AC3* do not attack intruder males. In the case of surgical removal, the diminution of maternal aggression is robust even in females who previously displayed the behavior (Bean and Wysocki, 1989; Stowers et al., 2002; Wang and Storm, 2011).

Parenting

The main olfactory epithelium appears to be necessary for maternal care of mouse pups. Maternal $ZnSO_4$ treatment decreases maternal nest-building pup survival similarly to, though less severely than, complete bulbectomy (Vandenbergh, 1973). When retrieving pups, female mice respond to a combination of olfactory and auditory cues (Smotherman et al., 1974). The olfactory cues are likely to be MOE-specific, since virgin females treated with $ZnSO_4$ or with lesions in the entorhinal and piriform cortex failed to show normal pup retrieval or normal responses to pups' ultrasonic vocalizations. In lactating

females, the lesions affected only the response to vocalizations and not retrieval success (Koch and Ehret, 1991). Mutation of *AC3*, which also abolishes MOE activity, also diminished nest-building and pup-retrieval (Wang and Storm, 2011). Although Vandenbergh found that bulbectomy led to more severe maternal deficits, the role of the VNO in maternal behaviors is thus far ill defined. Surgical removal of the VNO alone seemed to have no effect on maternal behavior (Lepri et al., 1985). Females mutant for *Trpc2* spend less time on the nest, particularly given an opportunity for exploration of a novel territory (Kimchi et al., 2007). However, it is not clear whether this is primarily a deficit in maternal care or an increase in exploratory behavior.

Behavior-promoting pheromones

In general, while it is clear that intraspecific chemosensation plays a crucial role in the control of sexually dimorphic behaviors in the mouse, pairing specific molecules with their effects has met with less success than might be expected. Some progress has been made for priming pheromones (Jemiolo and Novotny, 1993; Ma et al., 1998). As the behaviors above described, the clearest links have been found for VNO-stimulating peptide pheromones. ESP1, a peptide produced by the male lacrimal gland, has been linked to female receptivity (Haga et al., 2010). ESP1 is a member of a large family of sexually dimorphically and strain-specifically expressed peptides, many of which may also function as pheromones conveying information about individuals' sex and identity (Kimoto et al., 2007). The MUP family is another large subfamily of candidate peptide pheromones. The MUPs, members of the lipocalin family, are capable of binding small hydrophobic molecules and may thus act as pheromone carriers (Beynon and Hurst,

2004). However, the proteins have also been reported to act as pheromones in their own right, including individual MUPs capable of eliciting aggression (Chamero et al., 2007).

One explanation for the dearth of convincing identification of specific pheromones, particularly MOE-activating pheromones, may be that mixtures of molecules act in concert to provide a particular cue. As described above, odorants and receptors are not necessarily paired one-to-one in the MOE. A response to a mixture is even more likely in the case of chemosensory cues that are learned. Although some would separate the concept of a pheromone from that of a signature mixture (a chemical combination specific to an individual which is learned by a conspecific), most tests of a response to a putative pheromone will not distinguish between an innate response and a learned association.

Rather than approach the question of pheromonal control of behaviors from individual molecules, we used genetic tools to dissect the roles of the main and accessory olfactory systems and their interaction as a whole.

Chapter Two: VNO inhibition of male mating

Introduction

It is clear that chemosensory control is important for control of male-typical behaviors such as mating and aggression, and genetic tools have begun to elucidate the pheromonal control of these behaviors.

Males mutant for *Trpc2*, who thus lack VNO input, surprisingly show no deficit in mating with females, demonstrating that there is no requirement for a VNO-dependent positive signal to activate mating behavior. However, *Trpc2*$^{-/-}$ males show a lack of inter-male aggression. Moreover, these males attempt to mate with male intruders. They also emit ultrasonic vocalizations equally toward both males and females, implying that they have lost sex discrimination (Leypold et al., 2002; Stowers et al., 2002).

Males lacking *Cnga2* also lack inter-male aggression. They also show a severe deficit in mating behavior. This implies a positive requirement for the MOE for both behaviors. Moreover, although they interact with intruder females, they do not engage in anogenital chemoinvestigation (Mandiyan et al., 2005). This loss of chemoinvestigation might prevent pheromones from reaching the VNO. The lack of mating and aggression in *Cnga2*$^{-/Y}$ males could then be in whole or in part a consequence of the inability to detect VNO-specific pro-mating or pro-aggression cues.

Both the MOE and the VNO thus appear to be necessary for the appropriate behavioral output. The relative roles and interaction between these two chemosensory modalities is

still unclear. We used the above two knockout strains to investigate the interaction between the MOE and VNO in male mating behavior.

Experimental Procedure

Mice

Mice were housed as described previously and all studies were in accordance with IACUC protocols at UCSF (Mandiyan et al., 2005). Mice bearing targeted deletions of Cnga2 and Trpc2 have been described previously (Brunet et al., 1996; Leypold et al., 2002). Null mutants for Trpc2 and/or Cnga2 were maintained on a mixed 129/Sv and C57Bl/6J background. Mice were group-housed by sex at weaning and adult (10-30 weeks of age) animals were used for all studies. Control animals for all studies were WT or heterozygous for either Cnga2 or Trpc2.

Mice mutant for Cnga2 have difficulty feeding early in life. As described previously (Mandiyan et al., 2005), litters with Cnga2 null pups were trimmed to 4 - 5 pups. The dams were given peanut butter (Skippy Creamy) and high-caloric food pellets (LabDiet 5058) to allow the mutant pups to thrive. Pups were weaned 3 - 4 weeks after birth. Upon weaning, peanut butter and pre-wetted food pellets were provided every 2 - 3 days until 5 weeks after birth. The mice were subsequently given dry pellets on the cage floor for 5 days, after which the food pellets were only provided using the regular overhead feeders.

Behavioral testing

Testing was initiated ≥1 hour after onset of the dark cycle, and recorded and analyzed as described previously.

Males were isolated for at least 2 days and assays were performed with at least 1 day in between. Each male had 3 mating assays, 3 aggression assays, and then 3 more mating assays. All behavior assays were performed 1 to 8 hours after lights-out. The assays were recorded and scored blind to genotype.

For a mating assay, a primed intruder female was placed in the male resident's home cage for 30 minutes. For an aggression assay, a castrated wild-type male painted with intact wild-type male urine was placed in the male resident's home cage for 15 minutes.

Female intruders were wild-type females that had been ovariectomized and primed to be in estrus. Priming consisted of subcutaneous injection of 10 μg estradiol benzoate (EB) in 100 μL sesame oil 2 days before, 5 μg EB in 50 μL sesame oil 1 day before, and 50 μg progesterone in 50 μL sesame oil 4-8 hours before the assay.

Statistical Analysis

Quantification of behavioral data was performed blind to genotype. We performed the following tests of statistical significance. Categorical data was analyzed using a Fisher's exact test with a Holm-Bonferroni correction for multiple comparisons. Continuous data from multiple assays per animal was first averaged within each animal and then compared across groups. Data for a behavior includes only assays in which that behavior

occurred. For continuous data, we first tested the distribution of the data with Lilliefors' goodness-of-fit test of normality. Data not violating the assumption of normality were analyzed using parametric tests (One-way ANOVA with Tukey's post-hoc test) whereas data with a non-normal distribution was analyzed with the non-parametric Kruskal-Wallis (with Tukey's post-hoc test). We used an alpha level of 0.05 for all statistical tests.

Results

As expected, *Trpc2*$^{-/-}$ males mated with females fully as often as control males, while *Cnga2*$^{-/Y}$ males showed a decreased probability of mating, though mating was not completely absent. Interestingly, while *Cnga2*$^{-/Y}$;*Trpc2*$^{-/-}$ males also mated in fewer assays than control or *Trpc2*$^{-/-}$ males, they showed a slight increase in probability of mating compared to the single *Cnga2* mutant (Figure 2-1A). *Cnga2*$^{-/Y}$;*Trpc2*$^{-/-}$ also showed the same decrease in anogenital chemoinvestigation previously seen in the single *Cnga2* mutant (Mandiyan et al., 2005). They sniffed later and less per assay (Figure 2-1B-D). In assays with mating, however, mating proceeded normally regardless of genotype. In assays with mounting, intromission, or ejaculation, there was no difference in latency, number per assay, or duration of these behaviors (Figure 2-1B-D). (Only 2 of 8 *Cnga2*$^{-/Y}$ males mounted or intromitted and only one ejaculated, so it is impossible to draw conclusions about the pattern of mating in these animals. However, the assays were not obviously out of the normal range.)

When assays without mating were excluded, differences in sniffing patterns continued to hold, with double mutant and *Cnga2* single mutant males sniffing much less and later

than control or *Trpc2* single mutant males (Figure 2-2A), implying that normal sniffing is not required for mating. In fact, animals mutant for *Cnga2* were likely to begin mounting before they had sniffed, while animals wild-type for *Cnga2* never did (Figure 2-2B).

Figures

Figure 2-1

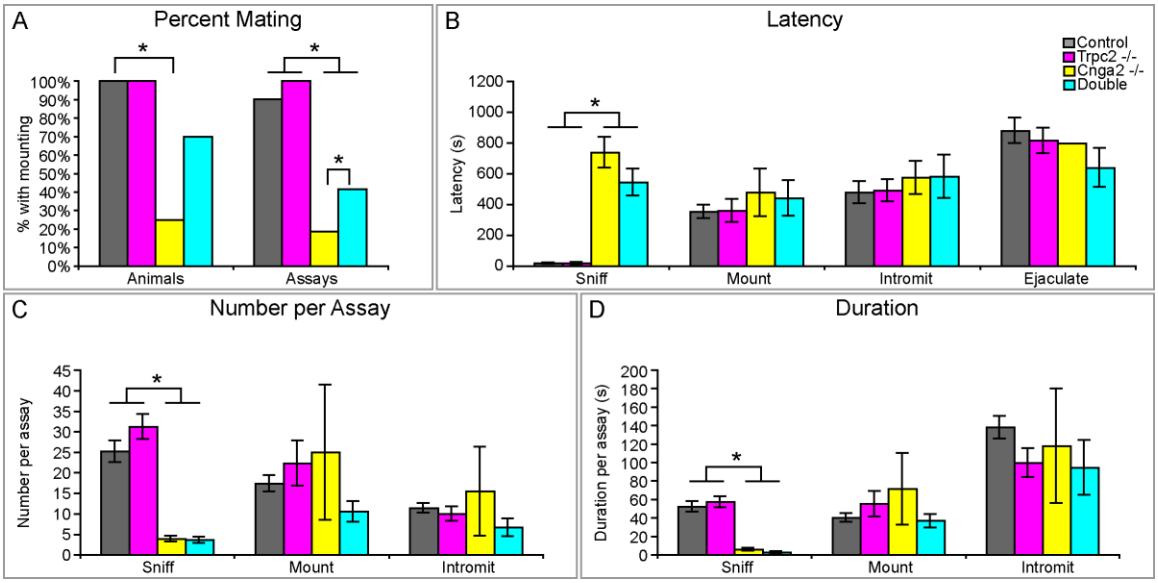

Figure 2-1 Olfactory input affects probability, not pattern of mating.

(A) Fewer *Cnga2*$^{-/Y}$ males mate than control males. Double mutant males mate in fewer assays than control or *Trpc2*$^{-/-}$ males, but more often than *Cnga2*$^{-/Y}$ males. Fisher's Exact Test with Holm-Bonferroni correction, N ≥ 5. (B) *Cnga2*$^{-/Y}$ and *Cnga2*$^{-/Y}$;*Trpc2*$^{-/-}$ males sniff later than control or *Trpc2*$^{-/-}$ males, but there is no significant difference in latency to mount, intromit, or ejaculate among those who do so. Sniff: Kruskal-Wallis P = 6.87*10^{-6}; Mount: ANOVA P = 0.74; Intromit: ANOVA P = 0.86; Ejaculate: ANOVA P = 0.40. (C) *Cnga2*$^{-/Y}$ and *Cnga2*$^{-/Y}$;*Trpc2*$^{-/-}$ males sniff less often per assay than control or *Trpc2*$^{-/-}$ males, but there is no significant difference in number of mounts or intromissions. Sniff: Kruskal-Wallis P =6.54 *10^{-6}; Mount: ANOVA P = 0.13; Intromit: Kruskal-Wallis P = 0.22. (D) *Cnga2*$^{-/Y}$ and *Cnga2*$^{-/Y}$;*Trpc2*$^{-/-}$ males spend less time sniffing per assay than control or *Trpc2*$^{-/-}$ males, but there is no significant difference in time spent of mounting or intromitting. Sniff: Kruskal-Wallis P = 6.75*10^{-6}; Mount: ANOVA P = 0.22; Intromit: ANOVA P = 0.35.

Figure 2-2

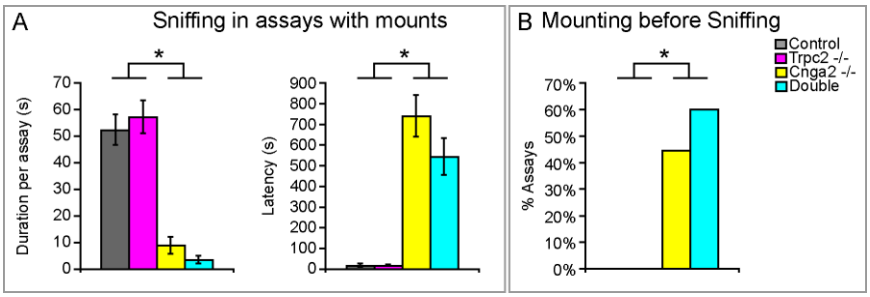

Figure 2-2 Sniffing is not required for mating.

(A) In assays with mounts, *Cnga2*$^{-/Y}$ and *Cnga2*$^{-/Y}$;*Trpc2*$^{-/-}$ males spend less time sniffing per assay and begin sniffing later than control or *Trpc2*$^{-/-}$ males. Duration: ANOVA P = 4.09 * 10^{-6}; Latency: Kruskal-Wallis P = 6.88 * 10^{-6}. (B) In assays with mounts, *Cnga2*$^{-/Y}$ and *Cnga2*$^{-/Y}$;*Trpc2*$^{-/-}$ males often begin mounting before having sniffed, while control and *Trpc2*$^{-/-}$ males never do. Fisher's Exact test with Holm-Bonferroni correction.

Conclusions

Although it might be expected that pheromonal input is necessary for male mating behavior, we found that abolition of pheromone sensing not only failed to eliminate mating, but actually resulted in more mating than the absence of MOE input alone. This suggests a model in which the VNO inhibits mating while the MOE activates it. Inhibition of mating by a chemosensory system would seem to be due to the presence of mating-inhibiting cues present when mating would be inappropriate, e.g. with a male; however, since loss of VNO activity increases mating with females in the context of MOE loss, it appears to be a tonic inhibition that is released in the presence of female cues. What modality is responsible for this release is unclear.

The probability of mating in the double mutant was intermediate between the two single

mutants, suggesting that the interaction between the two systems is not a simple upstream/downstream relationship but that both feed into a circuit determining the probability of initiating mating. The pattern of mounting, intromitting, and ejaculating was the same across genotypes, implying that pheromonal input is part of a switch, affecting the animal's choice to initiate mating, but not affecting the pattern itself. This pattern is likely encoded in brain regions downstream of the pheromone-influenced decision to mate.

In wild-type males, mating is nearly always preceded by intense anogenital chemoinvestigation (sniffing) of the female. This presumably allows access of pheromones, particularly non-volatile molecules, to the nose. This may be particularly important for pheromone access to the VNO. Yet, surprisingly, this sniffing was not absolutely required for mating. The VNO is required for inter-male aggression, and *Trpc2*$^{-/-}$ males have been reported to spend longer than controls sniffing males (Stowers and Logan, 2010). Taken together, this suggests that anogenital chemoinvestigation may be more important in aggression and that intense chemoinvestigation of females prior to initiating mating may serve partially or even primarily to confirm a lack of aggression-promoting cues. On the other hand, wild-type males show a preference for investigating female odors over male odors. These odors may be involved in the release of VNO mating inhibition as well as the separate positive effect of the MOE.

Chapter Three: Complex chemosensory control of reproductive behaviors in female mice

Introduction

Male mice employ odors processed by the main olfactory epithelium (MOE) and the vomeronasal organ (VNO) to display male-typical mating and aggressive behaviors. By contrast, the relative contributions of these two sensory epithelia to the display of female-typical behaviors are not well characterized.

Previous work on the role of pheromones on female physiology and behavior either has used physical/chemical methods to disrupt olfaction or has attempted with mixed success to find specific compounds responsible for effects on physiology or behaviors.

Prior to the availability of genetic knockouts, the roles of the MOE and VNO were differentiated by surgical and chemical methods. The main olfactory epithelium was ablated by irrigation of the nose with a solution of zinc sulfate. Depending on the protocol used, this method produces temporary (around 1 week due to regeneration of olfactory sensory neurons) peripheral anosmia in the majority of mice but often has a high rate of mortality and morbidity (McBride et al., 2003). Specific ablation of the vomeronasal organ has been done surgically, an approach that has produced conflicting results (Kimchi et al., 2007; Martel and Baum, 2009). Permanent removal of both MOE and VNO signals can be accomplished by the removal of the olfactory bulbs.

To better dissect the relative roles of the MOE and VNO and their interaction in female-typical behaviors, we used a genetic approach. Mice null for *Cnga2* or *Trpc2* essentially lack odor-evoked activity in the MOE and VNO, respectively (Brunet et al., 1996; Leypold et al., 2002; Stowers et al., 2002).

Priming effects of pheromones on female mice

Pheromones may affect reproductive behavior via priming or releaser effects. Both male and female pheromones have been implicated in the regulation of puberty and the estrous cycle, presumably by affecting the GnRH-releasing neurons in the hypothalamus.

Puberty acceleration and delay

Female mice complete puberty at approximately 6 to 8 weeks, with vaginal opening around 26 days (Caligioni, 2009; Silver, 1995). This process can be both accelerated and delayed by pheromones.

Exposure to male urine leads to earlier puberty (Vandenbergh effect, (Vandenbergh, 1969), an effect which appears to be mediated by the VNO, since it is TRPC2-dependent but independent of MOE ablation (Drickamer, 1986; Flanagan et al., 2011). Evidence for the specific male pheromones driving this effect, however, has been far from straightforward, and in fact results have sometimes directly contradicted each other. Various researchers have identified the puberty-accelerating compound(s) to be isobutylamine and isoamylamine (assessed by vaginal opening rather than uterine weight) (Nishimura et al., 1989), major urinary proteins (MUPs) but not the associated volatile compounds (Mucignat-Caretta et al., 1995), α- and β-farnesene, 2-sec-butyl-4,5-

dihydrothiazole, and 3,4-dehydro-exo-brevicomin but not MUPs (Novotny et al., 1999), or none of the previously identified pheromones (Flanagan et al., 2011).

In contrast, exposure to odors from group-housed females delay female puberty (Drickamer, 1982). This effect appears to be mediated at least in part by 2,5-dimethylpyrazine, an adrenal-mediated compound found in the urine of females (Jemiolo and Novotny, 1993; Novotny et al., 1986). It is not clear whether this delay of puberty is a function of the MOE, the VNO, or both, though 2,5-dimethylpyrazine appears to activate both systems, the former in a CNGA2-independent manner (Lin et al., 2007; Thompson et al., 2004).

Interestingly, odors from estrous, pregnant, or lactating females accelerate puberty (Drickamer, 1982; Drickamer and Hoover, 1979). This seems to be due to higher levels of certain ketones in the urine, coincident with a drop in 2,5-dimethylpyrazine (Jemiolo et al., 1989). The effect from estrus females may not be due to the same molecules as that from pregnant and lactating females, since the former is, like the acceleration by male pheromones, independent of the MOE, while the latter is MOE-dependent (Drickamer, 1986).

Estrous cycle acceleration and delay

Post-pubertal females have an estrous cycle of 4-5 days in isolation. Group-housed adult females show a longer average cycle due to exposure to adult female odors, a phenomenon known as the Lee-Boot effect (Van Der Lee and Boot, 1955). 2,5-dimethylpyrazine, the same pheromone implicated in puberty delay, is also capable of

mediating this effect (Ma et al., 1998). Surgical removal of the vomeronasal organ was sufficient to relieve the suppression of estrus in group-housed females (Reynolds and Keverne, 1979), implying that the Lee-Boot effect is VNO-dependent.

In contrast, disruption of the MOE by $ZnSO_4$ treatment (or of both MOE and VNO signaling via removal of the olfactory bulbs) lengthened the estrous cycle in isolated females (Vandenbergh, 1973). Since these females were exposed to neither male nor grouped female pheromones, it is not clear what causes this lengthening. It is possible that they respond to their own pheromones. Urine from other singly housed females does not appear to affect estrous cycles in isolated females, though urine from pregnant or lactating females extends estrus within each cycle (Hoover and Drickamer, 1979).

The Lee-Boot effect is counteracted by exposure to adult male odors (Whitten effect, (Vandenbergh, 2006; Whitten, 1956). As in the case of puberty, α- and β-farnesene, 2-sec-butyl-4,5-dihydrothiazole, 3,4-dehydro-exo-brevicomin, and MUPs have also been implicated in the Whitten effect, which also appears to be VNO-mediated (Jemiolo et al., 1986; Ma et al., 1999; Marchlewska-Koj et al., 2000).

Pregnancy block

If a female immediately post-mating encounters pheromones from a strange male, i.e. one not genetically identical to the male she just mated with, implantation is blocked and instead she returns to the estrus cycle and is capable of mating with the new male, an effect known as the Bruce effect (Bruce, 1959). This identification of individual remembered males is VNO-dependent, though *Trpc2*-independent, and depends on the

peptide ligands of the major histocompatibility complex (MHC) class I molecules (Kelliher et al., 2006; Leinders-Zufall et al., 2004).

Pheromone production

Interestingly, pheromonal input appears to play a role in the secretion of pheromones from females as well. Ablation of either the VNO or the MOE reduces production of the puberty-delaying pheromone from females, though the puberty-accelerating pheromones from males remain intact under either treatment. The puberty-accelerating effects are also intact after MOE ablation in estrous, pregnant, or lactating females (Drickamer, 1986; Lepri et al., 1985).

Releaser pheromones in female behaviors

Less is known about the releaser role of specific pheromones in female-typical mouse behaviors. However, there is evidence that both the MOE and VNO are necessary for the normal expression of these behaviors.

Female mating behavior

Destruction of the MOE via $ZnSO_4$ eliminates the female preference for intact male odors over those from castrate males (Keller et al., 2006a; Lloyd-Thomas and Keverne, 1982), while surgical removal of the VNO does not. On the other hand, surgical VNO removal, $ZnSO_4$, or olfactory bulbectomy are each sufficient to decrease female receptivity in mating tests (Keller et al., 2006a; Keller et al., 2006b; Thompson and Edwards, 1972). The VNO-dependent effect on receptivity has been proposed to be caused by exocrine

gland-secreting peptide 1 (ESP1), a molecule secreted by the male tear duct that activates the receptor V2Rp5 (Haga et al., 2010).

Maternal behaviors

The main olfactory epithelium appears to be necessary for maternal behaviors. Treatment with $ZnSO_4$ or mutation of the adenylyl cyclase required for MOE function (AC3) reduces maternal behavior such as nest building and pup-retrieval (Koch and Ehret, 1991; Vandenbergh, 1973; Wang and Storm, 2011). $ZnSO_4$ or olfactory bulbectomy also increases maternal neglect and thus litter death (Vandenbergh, 1973). Removal of the VNO, on the other hand, showed only slight decreases in maternal behavior and no effect on litter health (Lepri et al., 1985).

Mutation of either *AC3* or *Trpc2* diminishes maternal aggression (Kimchi et al., 2007; Wang and Storm, 2011), suggesting a requirement for both MOE and VNO input similar to that in inter-male aggression.

Male-typical mating behavior

Although wild-type females will mount other females (at lower rates than males do), they will never mount males. However, in the absence of VNO input, females not only mount females at male-like rates, but also mount males (as do males likewise mutant for *Trpc2*). This suggests a combination of two effects: an increase in male-like mating (not seen in the males) and the same loss of sex-discrimination seen in the males. These females do have higher testosterone levels than control females, which could explain the overall increase in male-like behavior. However, it is within the normal range for females and

below baseline male levels, so it is unclear whether this increase is enough to explain the increase in mating behavior (Coquelin and Desjardins, 1982; Kimchi et al., 2007).

Experimental Procedure

Mice

Mice were housed as described previously and all studies were in accordance with IACUC protocols at UCSF (Mandiyan et al., 2005). Mice bearing targeted deletions of Cnga2 and Trpc2 have been described previously (Brunet et al., 1996; Leypold et al., 2002). Null mutants for Trpc2 and/or Cnga2 were maintained on a mixed 129/Sv and C57Bl/6J background. Mice were group-housed by sex at weaning and adult (8-35 weeks of age) animals were used for all studies. Control animals for all studies were WT or heterozygous for either Cnga2 or Trpc2. WT stud males for testing female sexual behavior were either C57Bl/6J or hybrid 129/Sv and C57Bl/6J, while WT intruder males were 129/Sv.

Mice mutant for Cnga2 have difficulty feeding early in life. As described previously (Mandiyan et al., 2005), litters with Cnga2 null pups were trimmed to 4 - 5 pups. The dams were given peanut butter (Skippy Creamy) and high-caloric food pellets (LabDiet 5058) to allow the mutant pups to thrive. Pups were weaned 3 - 4 weeks after birth. Upon weaning, peanut butter and pre-wetted food pellets were provided every 2 - 3 days until 5 weeks after birth. The mice were subsequently given dry pellets on the cage floor for 5 days, after which the food pellets were only provided using the regular overhead feeders.

Vaginal opening and smears

Mice were checked for vaginal opening daily beginning at P18 - P20 until opening and were weighed the day of opening.

To assess estrous cycles, vaginal cytology was examined once daily for 5 weeks (Caligioni, 2009). Images were scored as being in diestrus, diestrus/proestrus transition, proestrus, proestrus/estrus transition, estrus, estrus/metestrus transition, metestrus, or metestrus/diestrus transition. The length of a cycle was defined as the number of days from a slide scored as being fully in estrus to the next, providing that at least one day out of estrus intervened.

Behavior Assays

Testing was initiated ≥1 hour after onset of the dark cycle, and recorded and analyzed as described previously.

To test for sexual receptivity, group-housed females were ovariectomized, and, subsequent to estrus induction with estrogen and progesterone, inserted singly into the home cage of a sexually experienced WT male for 30 min each in 4 assays, at least one week apart. 2 control females and 2 *Cnga2* mutant females only received 3 assays each. A female's receptivity in an assay was defined as the percentage of the male's mounts which proceeded to intromission. Assays in which the male did not mount were discarded from analysis.

To test pup retrieval, experimental females were impregnated by a WT male and singly

housed before parturition. 3 times between 1 and 9 days after parturition, the dam was removed briefly from the cage and the pups placed in a corner of the cage floor away from the nest. The dam was returned to the cage and her pup retrieval ability was tested for 15 min.

Following pup retrieval assays, we tested maternal aggression 3 times between 7 and 15 days after parturition. Pups were removed and a group-housed adult WT male intruder was inserted into the cage for 15 min. The pups were returned to the mother at the end of each assay.

To test female mounting of males, sexually naïve females were isolated for 2-7 days and then tested 3 times with a WT group-housed vasectomized male intruder for 15 min each. Experimental mice were exposed to intruders they had not encountered previously, and each assay was separated by ≥ 2 days.

Statistical Analysis

Quantification of behavioral data was performed blind to genotype. We performed the following tests of statistical significance. Categorical data was analyzed using a Fisher's exact test with a Bonferroni correction for multiple comparisons. Continuous data from multiple assays per animal was first averaged within each animal and then compared across groups. Data for a behavior includes only assays in which that behavior occurred. For continuous data, we first tested the distribution of the data with Lilliefors' goodness-of-fit test of normality. Data not violating the assumption of normality were analyzed using parametric tests (One-way ANOVA for ≥ 3 groups with Tukey post-hoc test,

Student's t test for 2 groups) whereas data with a non-normal distribution was analyzed with the non-parametric Kruskal-Wallis (with Tukey's post-hoc test) or Kolmogorov-Smirnov test. We used an alpha level of 0.05 for all statistical tests.

Results

The MOE and VNO can potentially control female behaviors both indirectly, by affecting the animal's endocrine status and directly by acute control of behavior. Since female mating is dependent on the estrus cycle, we examined the onset of puberty and the subsequent timing of the estrus cycle in mice mutant for *Cnga2* or *Trpc2*.

Onset of puberty is independent of VNO input

Previous results have found a role for male and female pheromones in regulating the timing of puberty in females. The acceleration of puberty by male or estrous female odors has been reported to be VNO-dependent, while the role of the MOE or VNO in puberty delay is unclear.

In females that were group-housed with other females upon weaning, we found an effect of genotype on the age of vaginal opening, though no group showed significantly earlier or later opening (Figure 3-1A). The trend appeared to be toward slightly later opening in females mutant for *Cnga2*, regardless of VNO status. These females were group-housed with other females (of varying ages) after weaning, but were not rigorously protected from male odors. Before weaning, they were all exposed to paternal odors.

Animals lacking *Cnga2* are known to have difficulty feeding early in life, and in fact at

the age of vaginal opening *Cnga2*$^{-/-}$ females are significantly smaller than control females (Figure 3-1B). Undernutrition can delay puberty (Hansen et al., 1983), and the data presently do not exclude the possibility that the delay in vaginal opening results, at least in part, from the lower body weight in these females.

No requirement for MOE or VNO for estrous cycle

Research on the Lee-Boot and Whitten effects suggests that loss of MOE or VNO input would change the length of the estrous cycle in mice as well as other mammals. The VNO appears to be required for both effects, implying that the direction of the change in *Trpc2* mutants would depend on the pheromonal surroundings. The role of the MOE is unclear, although in isolated females, MOE destruction lengthened the estrous cycle.

Contrary to our expectations, we saw no significant effect of the loss of either *Cnga2* or *Trpc2* on estrous cycle length (measured from estrus to estrus) or on the percentage of females coming into estrus within the first 5 days of observation (Figure 3-2:A-B).

Redundant control of sexual receptivity

Having seen no requirement for the MOE or VNO for normal endocrine physiology, we investigated the role of these systems for acute control of behavior. We examined sexual receptivity in females that were ovariectomized and then supplemented with hormones to recapitulate estrus. Compared to controls, *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ females showed a significant decrease in receptivity, measured as the percentage of mounts by the male that proceeded to intromission. However, the MOE and VNO appear to play a redundant role in female receptivity, since neither mutation alone was enough to cause a significant loss of

receptivity (Figure 3-3C). This implies that females use pheromones to identify potential mates, though they can access that information vie either the MOE or the VNO.

The MOE is required for pup retrieval

Mice with pups show maternal care, keeping the pups in a nest for safety and warmth, nursing them, and licking them. One measure of maternal care of pups is retrieval of pups that have been removed from the nest, which requires the recognition of pups. In assays in which 3 pups were placed across the cage from the nest, mutation of *Cnga2* severely decreased the probability of the mother even picking up a pup, with a corresponding decrease in the probability of returning all 3 pups to the nest. A further effect of *Trpc2* deletion appeared only in the context of the *Cnga2* mutation, while $Trpc2^{-/-}$ retrieved pups as well as controls (Figure 3-3:A-B). Even those $Cnga2^{-/-}$ females who did pick up pups were slower than control or $Trpc2^{-/-}$ females (Figure 3-3C). ($Cnga2^{-/-};Trpc2^{-/-}$ females picked up pups too rarely to analyze latencies.) Failure to pick up or retrieve pups is not due to avoidance of the pups; all females came in physical contact with pups and there was no significant difference between genotypes in latency to do so (Figure 3-3D). This suggests that females need olfactory cues to identify pups as pups, even in the presence of auditory and tactile cues.

MOE and VNO both required for maternal aggression

Although female mice usually do not show aggression toward males, nursing females will attack unfamiliar intruder mice to protect their pups (Gandelman, 1972). As in the case of inter-male aggression (Leypold et al., 2002; Mandiyan et al., 2005; Stowers et al.,

2002), mutation of either *Cnga2* or *Trpc2* was sufficient to completely abolish this maternal aggression; while 92% of controls attacked intruders, *Trpc2*$^{-/-}$, *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$, and *Cnga2* $^{-/-}$ females never did (Figure 3-3:E-F). Thus, it seems that aggression requires positive inputs from both the MOE and VNO.

The MOE is not required for male-like mounting of males

Trpc2$^{-/-}$ females show male-like mounting toward intruder males, which is not seen in WT females (Kimchi et al., 2007). We found that this aberrant mounting was not dependent on CNGA2, with *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ females mounting intruder males at the same frequency as *Trpc2*$^{-/-}$ females (Figure 3-4:A). In fact, double mutant females began mounting sooner and mounted more times per assay than the single mutants (Figure 3-4:B-C). The accelerated onset of mounting in the double mutant females may reflect a decrease in anogenital chemoinvestigation ("sniffing"). *Trpc2*$^{-/-}$ females sniff sooner and more often than double mutants do (Figure 3-4:D-F). Neither control females nor *Cnga2*$^{-/-}$ females ever mounted males.

Figures

Figure 3-1

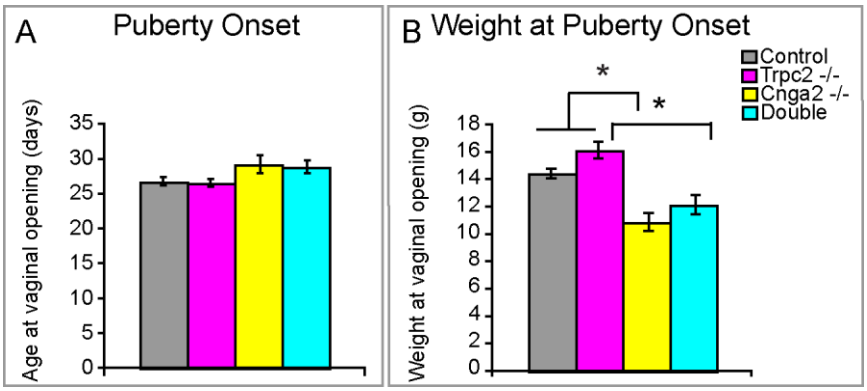

Figure 3-1 Pheromonal input is not necessary for normal puberty onset.

(A) Loss of *Trpc2* has no effect on age of vaginal opening. Mean ± S.E.M. Kruskal-Wallis: $\chi^2 = 8.61$, P = 0.035, no significant pairwise differences between groups. N ≥ 7. (B) Lack of MOE function retards growth; *Cnga2*$^{-/-}$ females begin puberty at lower weights. Mean ± S.E.M. ANOVA: F = 23.5, P = 3.1 * 10^{-5}. N ≥ 7.

Figure 3-2

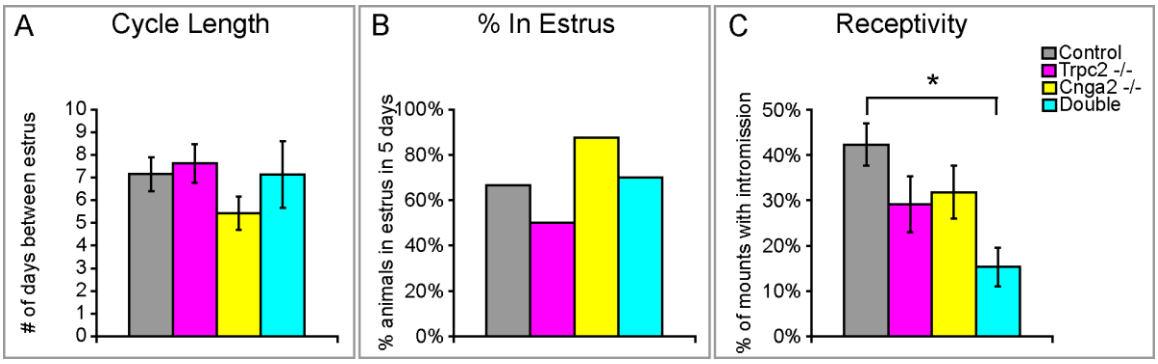

Figure 3-2 Intact estrous cycle but decreased receptivity in *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ females.

(A) The number of days between vaginal smears showing estrus is not significantly different from controls in females mutant for *Cnga2*$^{-/-}$ and/or *Trpc2*$^{-/-}$. Mean ± S.E.M. Kruskal-Wallis: $\chi^2 = 2.61$, P = 0.55. N ≥ 8. (B) In the first 5 days of observation, most females were in estrus on at least one day, with no significant effect of genotype. Fisher's Exact Test: P > 0.1 N ≥ 8. (C) *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ females showed a significant decrease in sexual receptivity as measured by the percent of mounts from the male that proceeded to intromission. Mean ± S.E.M. One-way ANOVA with Tukey's Post-Hoc: F=3.41, P = 0.024. N ≥ 8.

Figure 3-3

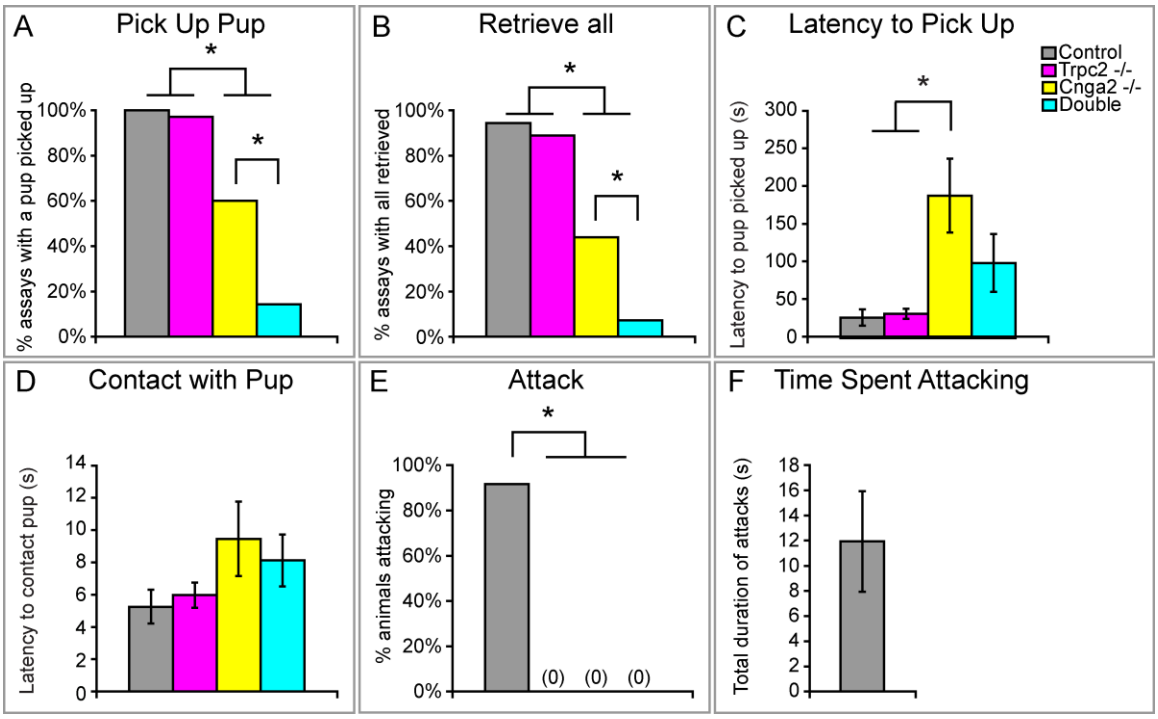

Figure 3-3 Diminished maternal behaviors in female olfactory mutants.

(A) *Cnga2*$^{-/-}$ and *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ females are less likely to pick up a pup that's been removed from the nest, with the double mutants even less likely than the *Cnga2*$^{-/-}$; single mutants. Fisher's Exact Test with Bonferroni correction. N ≥ 9. (B) *Cnga2*$^{-/-}$ and *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ females are less likely to return all three pups to the nest within 15 minutes, with the double mutants even less likely than the *Cnga2*$^{-/-}$; single mutants. Fisher's Exact Test with Bonferroni correction. N ≥ 9. (C) Even *Cnga2*$^{-/-}$ females who did pick up pups were slower to do so than control or *Trpc2*$^{-/-}$ females. Mean ± S.E.M. Kruskal-Wallis: 12.16, P = 0.0069. (D) There were no significant differences between genotypes in latency to make physical contact with a pup. Mean ± S.E.M. ANOVA: F = 1.32, P = 0.28, N ≥ 9. (E) While almost all control nursing females attacked a male intruder, *Trpc2*$^{-/-}$, *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$, and *Cnga2*$^{-/-}$ females never did. Fisher's Exact Test with Bonferroni correction. N ≥ 4. (F) Control females spent a total of 11.9 ± 3.3 s per assay attacking male intruders. Mean ± S.E.M.

Figure 3-4

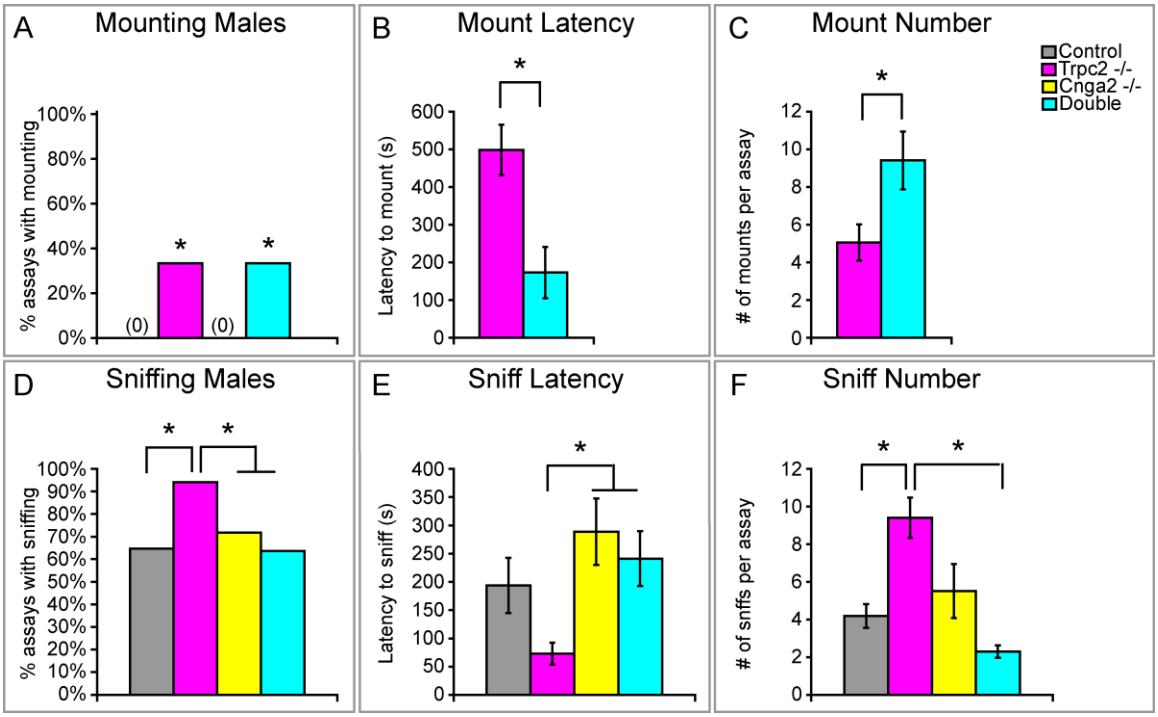

Figure 3-4 Mounting of males by *Trpc2*$^{-/-}$ females does not require *Cnga2*.

(A) *Trpc2*$^{-/-}$ and *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ females mount males with the same frequency, while controls and *Cnga2*$^{-/-}$ single mutants never do. Fisher's Exact Test with Bonferroni correction. N ≥ 11. (B) *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ females initiate mounting sooner than *Trpc2*$^{-/-}$ females. Mean ± S.E.M. t-test: P = 0.025. (C) In assays in which the resident female mounts the intruder male, *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ females mount males more times per 15 minute assay than *Trpc2*$^{-/-}$ females. Mean ± S.E.M. t-test: P = 0.006. (D) *Trpc2*$^{-/-}$ females are more likely to engage in anogenital chemoinvestigation ("sniffing") with male intruders than the other genotypes. Fisher's Exact Test with Bonferroni correction. N ≥ 11. (E) *Trpc2*$^{-/-}$ females sniff male intruders sooner than *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ or *Cnga2*$^{-/-}$ females. Mean ± S.E.M. Kruskal-Wallis: $\chi^2 = 14.5$, P = 0.0023 (F) *Trpc2*$^{-/-}$ females sniff male intruders more often per assay than control or *Cnga2*$^{-/-}$;*Trpc2*$^{-/-}$ females. Mean ± S.E.M. Kruskal-Wallis: $\chi^2 = 19.65$, $P = 2.0*10^{-4}$

Conclusions

Pheromonal control of female-typical behaviors is complex, with each behavior having a different requirement for MOE and/or VNO input. This is the case even when the task appears superficially similar. For instance, identifying a male as a potential mate (triggering receptivity) requires either the MOE or VNO, but not both, and a low level of receptivity is independent of pheromonal input. In contrast, identifying a male intruder as a target for attack in maternal aggression appears to have stricter requirements, with both MOE and VNO function required for any aggression. Interestingly, this is true of inter-male aggression as well in spite of the many differences between these behaviors. Not only are the internal cues different, but the signals identifying the target of the aggression cannot be identical even though the required sensory modalities are the same. In lactating females, female intruders are capable of eliciting maternal aggression (Gandelman, 1972), while wild-type males do not attack female intruders.

In wild-type females, a male intruder is also identified as an inappropriate target of male-like mating behavior, and this is entirely dependent on the VNO. Surprisingly, in the absence of VNO function, this mating behavior becomes independent of MOE input. This implies that the VNO normally inhibits mating behavior and that normal mounting represents a release of this inhibition. In male mating with females, the VNO also appears to have an inhibitory role. However, even in the context of the *Trpc2* mutation, the *Cnga2* mutation causes a slight decrease in the probability of mating, with the double mutant mounting more than the *Cnga2* single mutant and less than the *Trpc2* single mutant (Chapter Two). The fact that the double mutant females mount males at the

same rate as the single *Trpc2* females distinguishes the control of this behavior from that of males mounting females, in which the double mutant is intermediate between the *Cnga2* and *Trpc2* single mutants. This implies that males require some positive MOE-dependent signal from females to reach wild-type (and *Trpc2*) mating rates. Sexual dimorphisms in odor processing may play a role in these differential responses to pheromonal cues, though thus far the evidence for sexual dimorphism in the MOE has been scanty (Martel and Baum, 2007; Shiao et al., 2012). On the other hand, direct comparisons between these two experiments may be misleading. It is necessary to examine whether the MOE plays a role in female mounting of females.

Taken together, this suggests a model in which the VNO provides tonic inhibition of male-like mating in both males and females. In the presence of a female, this inhibition is relieved. This release of inhibition could come from the MOE or VNO, or another modality such as auditory cues.

Auditory cues from pup vocalizations and physical contact with pups together are insufficient to trigger the retrieval of pups, suggesting that odor cues are necessary for the identification of pups as targets of retrieval. Interestingly, the VNO appears to play a redundant role that, at least in the laboratory context, only appears in the context of a lack of MOE function.

Unexpectedly, we saw little pheromonal dependence of female endocrine physiology, with a small effect (probably of the MOE) on puberty onset and no role in the estrous cycle. Females were housed with their father until weaning and then group-housed at weaning with other females. They should have had access to female puberty/estrus-

delaying pheromones and may have had access to male puberty/estrus-accelerating pheromones. We did not test the effects of deliberately withholding or adding pheromone cues, which might have revealed covert differences in endocrine control.

Thus female-typical behaviors require chemosensory information, but the role of the two systems depends on the behavior examined. Future work should investigate the connections between the olfactory input and the downstream circuits involved in these behaviors. In particular, in behaviors such as aggression, it is unclear how information from the MOE and VNO is integrated to produce a single behavioral output.

Chapter Four: Analysis of wild-type male mating

Introduction

Male mouse mating pattern

Male mouse mating behavior has a stereotyped pattern that can remain intact even when the probability of initiating mating is perturbed (Juntti et al., 2010). Upon encountering a receptive female, the male investigates her, particularly the anogenital region. He follows the female around, sniffing and rooting. He begins to mount the female from behind, with the forepaws on her flanks, thrusting rapidly. There may be several mounts and dismounts before he first penetrates the vagina. Intromission (penetration) can be identified when the pattern of thrusting slows and becomes noticeably deeper. Ejaculation generally does not take place until after many mount-intromission-dismount repetitions. Upon ejaculation, the male freezes in place on the female for as much as 25 seconds and generally falls over (Hull and Dominguez, 2007; McGill, 1962). Ejaculation is followed by a refractory period lasting minutes to hours (depending on strain) in which the male does not mate (Mosig and Dewsbury, 1976).

Genetic control of mating pattern

While the sequence of behavior is consistent across strains, many quantitative measures differ between inbred strains, demonstrating that they are genetically hardwired. Mount and ejaculation latencies, for example, both differ over five-fold between C57BL/6J and C3H/HeJ (Levine et al., 1966; McGill, 1962; McGill and Blight, 1963; Mosig and

Dewsbury, 1976). More recently, our lab has identified a gene, *Sytl4*, whose deletion perturbs only selected parameters of male mating (Xu et al., 2012).

Hormonal control of male mating

Testosterone is necessary for normal mating behavior. In mice, sex is determined by the presence of absence of the *Sry* gene on the Y chromosome. In males, the expression of *Sry* directs the bipotential gonad to develop into testes, which then produce testosterone which masculinizes secondary sex characteristics and behaviors (Harley et al., 2003).

Testosterone plays two roles in masculinizing behavior. In development, the testes produce a perinatal surge of testosterone (McCarthy, 2008). (At this age, the ovaries of females are quiescent, so they experience neither testosterone nor estrogen.) This testosterone, via local conversion to estrogen, masculinizes brain circuits and prepares them to respond in a male-like manner later in life (Wu et al., 2009).

Surprisingly, however, the developmental masculinization of brain circuitry does not appear to apply to male mating behavior. Apart from ejaculation, which requires male anatomy, female brains are perfectly capable of directing male-like mating behavior in response to a male-like hormonal milieu. At puberty, testes again produce high levels of testosterone, while female ovaries produce estrogen and progesterone. As in development, testosterone is locally converted to estrogen in the brain. A variety of genetic and pharmacological manipulations has demonstrated that, in both adult males and adult females, estrogen and testosterone act additively to generate male levels of male-like mating behaviors. Castrate males or females show little or no male mating

behavior (Edwards and Burge, 1971; McGill and Tucker, 1964). Wild-type females (estrogen only), brain-specific AR null males (estrogen only), and aromatase null males (testosterone only) mate with females, but at lower levels than wild-type males, while females given testosterone in adulthood mate at wild-type male levels (Edwards and Burge, 1971; Honda et al., 1998; Juntti et al., 2010; Wu et al., 2009). Males mutant for estrogen receptors also lack mating behavior (Ogawa et al., 2000).

However, data associating levels of testosterone with mating behavior have been contradictory. In wild-type male mice, luteinizing hormone (LH), which stimulates testosterone synthesis, is secreted in an irregular pulsatile pattern. This pattern causes periodic peaks in testosterone levels several times a day during which serum testosterone levels rise to as much as 20 times baseline levels and then return to baseline over the course of an hour (Coquelin and Desjardins, 1982). Exposure to female odors or mating experience has been reported to stimulate one of these pulses (Gleason et al., 2009). Some reports correlate increased testosterone with decreased latency to begin mating (James and Nyby, 2002). Interestingly, average testosterone levels in different strains is actually negatively correlated with that strain's mating behavior (Batty, 1978).

Even at baseline levels (1-2 ng/mL), serum testosterone levels are above the binding affinity for AR (Wilson and French, 1976), though this does not necessarily ensure that local levels of testosterone (and estrogen) in the brain will be high enough to fully activate behaviors.

Detailed analysis of male mating behavior

In our lab we want to better understand how brain circuits are programmed by genes to control these behaviors. A better understanding of the wild-type behavior patterns that we perturb in our experiments will improve our interpretations and allow us to better understand the circuits that create them.

Early papers describing the pattern of male mating reported a variety of quantitative parameters, including latencies to mount, intromit, and ejaculate and the durations of individual behaviors. With the advent of computerized data analysis, we should be able to produce a more detailed analysis of these patterns.

In order to extract more information about wild-type mating patterns than we had in previous experiments, we combined data from control animals from several published experiments into one data set. This larger data set gives greater statistical power to our analysis than we could have from a single experiment.

Experimental Procedure

Existing data set

Data from control mating assays in several previously published experiments were pooled to create a larger data set. In each assay a wild-type female, hormonally primed to be in estrus, had been put into the home cage of an isolated C57BL/6J intact male and recorded for 30 minutes. The video had been then manually scored blind to genotype

using a MATLAB-based program for manual scoring of behaviors developed for our lab (Juntti et al., 2010; Xu et al., 2012). The combined data set consists of 124 assays from 42 animals. 3 animals had 4 assays each, 35 had 3 assays, 3 had 2 assays, and 1 had only one assay.

Assay logs included data for four key behaviors in male mating performed by the resident male: anogenital chemoinvestigation ("sniffing"), mounting (exclusive of intromission), mounting with intromissive thrusting ("intromission"), and ejaculation. Logs consisted of start and end times for each bout of sniffing, mounting, and intromission and a single time point for an ejaculation. Intromission by definition was preceded by a mount, and ejaculation was always preceded by intromission.

Statistical analysis

Categorical data was analyzed using a Fisher's exact test with a Bonferroni correction for multiple comparisons. For continuous data, we first tested the distribution of the data with Lilliefors' goodness-of-fit test of normality. Data not violating the assumption of normality were analyzed using the parametric Student's t test, whereas data with a non-normal distribution was analyzed with the non-parametric Kolmogorov-Smirnov test (KS-test). Testing for assay independence was done by permutation test. Cochran's q test was used to test the effect of experience on frequencies of behaviors (MATLAB script by Jos van der Geest, MATLAB Central File Exchange). We used an alpha level of 0.05 for all statistical tests.

Hour-long mating assays

Mice were housed as described previously and all studies were in accordance with IACUC protocols at UCSF. Group-housed C57BL/6J males were isolated at approximately 8 weeks old. 60 minute mating assays were performed 5, 8, and 12 days after isolation.

During the assay, primed intruder females were placed in the male resident's home cage for 60 minutes. All behavior assays were performed 1 to 8 hours after lights-out. The assay was recorded and scored blind to individual. Statistical analysis was performed as above.

Intruders were C57BL/6J females that had been ovariectomized and primed to be in estrus. Priming consisted of subcutaneous injection of 10 μg estradiol benzoate (EB) in 100 μL sesame oil 2 days before, 5 μg EB in 50 μL sesame oil 1 day before, and 50 μg progesterone in 50 μL sesame oil 4-8 hours before the assay.

Testosterone homogenization and mating assays

Group-housed C57BL/6J males were castrated or sham castrated at 8-9 weeks old. Beginning 2 weeks after surgery, they were injected subcutaneously every other day, castrated males with 100 μg testosterone propionate (TP) in 50 μL sesame oil, sham castrated males with sesame oil alone. Treatment continued until all assays were complete. 20 or 22 days after surgery, males were isolated. 30 minute mating assays (as described for 60 minute assays) were performed 28, 32, and 34 days after surgery, 4 to 8

hours after treatment, 1 to 8 hours after lights-out. The assay was recorded and scored blind to individual and treatment. Statistical analysis was performed as above.

Results

Variability and patterning in male mating

The pooled wild-type data set consisted of 124 mating assays from 42 different males. Although male residents were genetically identical, behavior in mating assays varied widely between assays (Figure 4-1A). While all assays included sniffing, only 25% reached ejaculation during the assay, and 35% did not even mount (Figure 4-1B).

For the purposes of statistical analysis, all assays were treated as independent, even though there were multiple assays from individual animals. In fact, animals that had 3 assays in the data set animals were as likely to mount, intromit, or ejaculate in one or two assays as in none or all three. This was not because animals were more likely to mate in later assays; experience did not affect the probability of these behaviors (Figure 4-1D). However, assays were not independent of animal identity; animals were more likely to mount or intromit consistently (all the animal's assays or none) than would be predicted if assays were truly independent of animal identity (Figure 4-1E, data for intromission (permutation test: $P = 0.012$) and ejaculation ($P = 0.25$) not shown).

In spite of assay-to-assay variability, a general pattern of male mating exists. In a typical successful mating, early bouts of sniffing are succeeded by mounts. Mounts begin to be followed by intromissions, though there may still be mounts that do not transition to

intromission. After several bouts of intromission, the male ejaculates. After ejaculation, there is a refractory period without mounting and with little or no sniffing (Figure 4-1A). This pattern can be assessed quantitatively: in assays with at least one mount, most sniffing occurs before the first mount (Figure 4-2A), although mounting usually begins in the first third of the assay (Figure 4-1C). Once mounting begins, sniffing bouts are shorter and farther apart (Figure 4-2A). In assays with at least one intromission, most mounting occurs after the first intromission. Once intromission begins, mounts (without intromission) are shorter, probably because most of them quickly transition to intromission, and closer together (Figure 4-2B).

Predictors of ejaculation

In the 66 assays in which the male proceeded so far as to intromit, just under half ejaculated by the end of the assay (Figure 4-1B). What predicts ejaculation or non-ejaculation? Several parameters differ between assays with ejaculation and those without (but with intromission). Assays with ejaculation have less sniffing, in number and length of bouts, and fewer episodes of mounting than those without ejaculation, but mounting and intromitting begin earlier (Figure 4-3A-C). On the other hand, there is no significant difference in intromission number or in mount or intromission bout duration, and assays with and without ejaculation show a similar short latency to begin sniffing (Figure 4-3A-C). The mean inter-state interval (ISI, time between bouts) of sniffing is shorter in assays without ejaculation, but the ISIs do not differ in mounting or intromission (Figure 4-3D).

Mount latency is a key predictor of ejaculation

Since we know that there is a sequence to mating in which most sniffing is performed before the first mount and intromitting necessarily follows a mount, a shorter mount latency could explain both decreased sniffing and later intromission.

In fact, differences in sniffing essentially disappear when controlling for differences in mount latency. Mean duration of sniffing bouts before or after the first mount and sniffing ISI before the first mount do not predict ejaculation (Figure 4-4A). The sole remaining difference in sniffing, the ISI of sniffing after mounting begins, can be explained by decreased sniffing during the refractory period after ejaculation (Figure 4-4B). Thus, if we consider ejaculation to mark the end of an assay, differences in sniffing between ejaculators and non-ejaculators appear entirely dependent on mount latency.

The lower intromission latency in ejaculators also appears to be explained by the lower mount latency, since the time between the first mount and the first intromission does not differ significantly between the two groups (Figure 4-4C), though there are more mounts before the first intromission in assays without ejaculation (Figure 4-4E).

Finally, although lower mount and intromission latencies combined with similar ISIs would suggest that ejaculators would have more mounts and intromissions than non-ejaculators, they in fact have fewer mounts and no difference in intromission number (Figure 4-3A). This result, however, is explained by the post-ejaculation refractory

period. Treating ejaculation as the end of an assay gives ejaculators less time to mount and the same amount of time to intromit as non-ejaculators (Figure 4-4D).

It seems, therefore, that of the parameters scored in these assays, a shorter mount latency is the key predictor of ejaculation within the 30-minute assay. Moreover, if the average time from first intromission to ejaculation in assays with ejaculation (657s ± 57s, Mean ± S.E.M.) applies to assays without ejaculation (latency to intromit 1009s ± 88s), the mean time to ejaculate would be nearly 28 minutes, very close to the end of the assay. This suggests that the difference between assays with and without ejaculation may be simply explained by the 30 minute cutoff of the assay.

In hour-long assays, preliminary evidence suggests that this may indeed be the case. Although with the current low sample size there was no significant increase in the probability of ejaculating when assays were extended, 3 of 4 animals that mounted but did not ejaculate in the first 30 minutes did in fact ejaculate in the remaining 30 minutes (Figure 4-4F). If this is representative of a larger group, this suggests that most animals that mount and intromit but do not ejaculate in a 30-minute assay would proceed to ejaculation if provided more time and that a high latency to mount not only predicts but explains a failure to ejaculate. The causes underlying the variability in latency to begin mating, however, remain to be explained.

Variability is not explained by testosterone

Testosterone is necessary for the full expression of male-typical behavior (Juntti et al., 2010) and may vary widely between assays, even within a single animal. In males,

luteinizing hormone (LH), which stimulates testosterone production, is released from the pituitary in irregular pulses. As a result, serum testosterone levels in male mice peaks over the course of around an hour every few hours, during which testosterone levels rise to over 10-fold above baseline (Coquelin and Desjardins, 1982). Thus in addition to any individual variation, it is impossible to control for an assay's timing relative to a testosterone peak. It is possible that differences in testosterone levels are responsible for variation in mating success (James and Nyby, 2002).

Since measurements at the end of an assay may not adequately reflect the hormonal milieu before and during the assay, it is necessary to control for testosterone levels by treating males to equalize these levels. Castrating males and treating with testosterone to decrease variability in levels increases the likelihood of mounting but has no significant effect on the likelihood of intromission and leaves the probability of ejaculation unchanged (Figure 4-5A). Males castrated and treated with testosterone initiate behaviors with the same latency as control males and showed no difference in number or length of the behaviors scored in the original data set (Figure 4-5B-D). However, interestingly, males castrated and treated with testosterone show a change in grooming, a behavior not included in the original mating data set. Treatment decreased the number and mean duration of grooming bouts (Figure 4-5C-D).

Figures

Figure 4-1

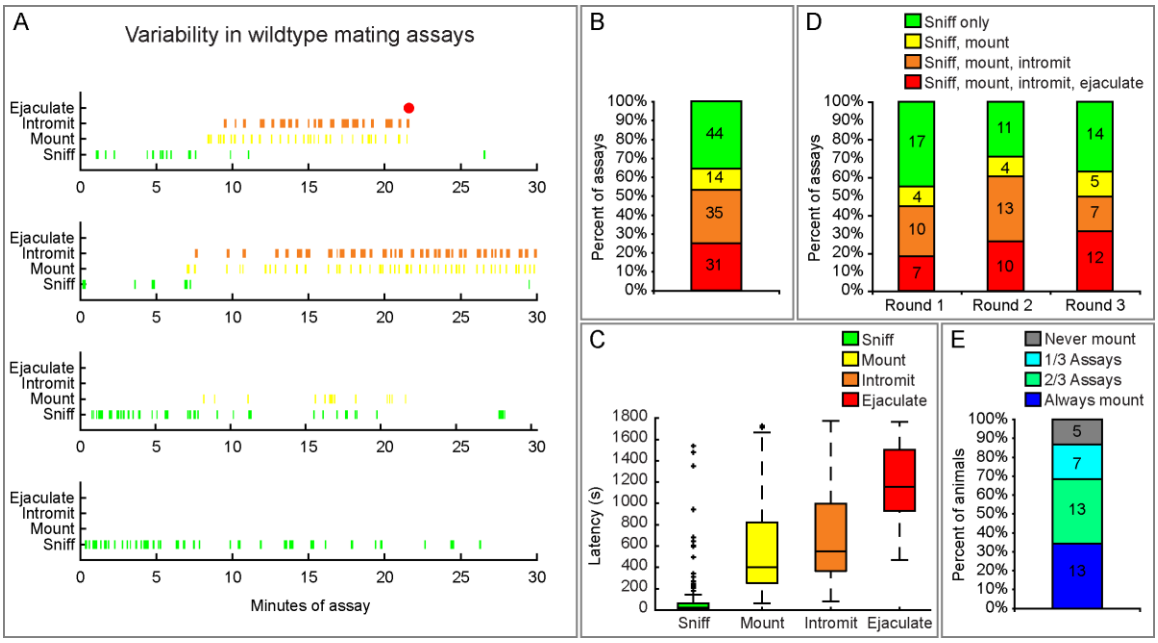

Figure 4-1 Wild-type males show variability in mating assays.

(A) Raster plots of 4 sample mating assays from data set. (B) Summary of 124 assays for wild-type mating data set. Only 25% of assays include ejaculation, while 35% do not even contain mounting. (C) Latencies to begin behaviors in assays with those behaviors (sniff N = 124, mount N = 80, intromit N = 66, ejaculate N = 31) (Boxes show median and upper/lower quartiles.) (D) Success in mating is independent of mating experience. In animals whose first, second, and third mating assays were all in the data set, there was no effect of round number on likelihood of mounting, intromitting, or ejaculating (Cochran's Q test, N = 38 animals: mount Q = 2.70, P = 0.26; intromit Q = 2.55, P = 0.28; ejaculate Q = 1.90, P = 0.39). (E) Mounting in assays was not independent of animal identity. In animals whose first, second, and third mating assays were all in the data set, more animals mounted in 0/3 or 3/3 assays (vs. 1/3 or 2/3) than would be expected based on overall rates of mounting if assays were independent of animal identity (permutation test: P = 0.009, N = 38 animals).

Figure 4-2

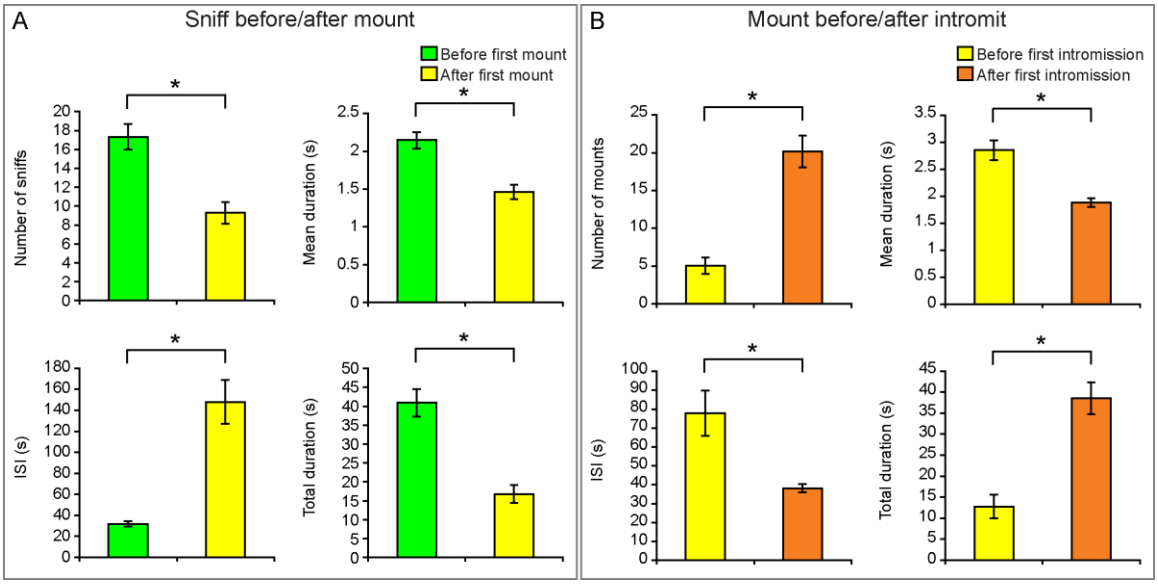

Figure 4-2 Temporal sequence of sniffing, mounting, and intromission

(A) In assays with at least one mount, most sniffing occurs before the first mount. (KS-test: number P = 4.8 * 10^{-4}, total duration P = 1.6 * 10^{-5}). Once mounting begins, sniffing bouts are shorter (t-test: P = 3.2 * 10^{-5}) and farther apart (KS-test: P = 2.8 * 10^{-10}). Mean ± S.E.M., N = 80 assays. (B) In assays with at least one intromission, most mounting occurs after the first intromission (KS-test: number P = 6.7 * 10-8, total duration P = 2.9 * 10-7). Once intromission begins, mounts (without intromission) are shorter (KS-test: P = 5.3 * 10-5) and closer together (KS-test: P = 4.5 * 10-6). Mean ± S.E.M., N = 66 assays.

Figure 4-3

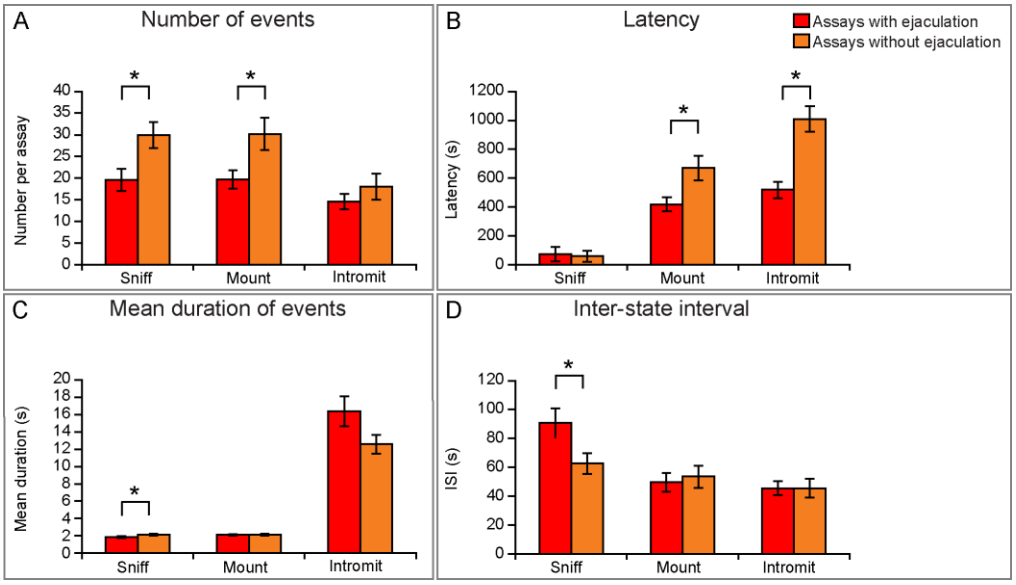

Figure 4-3 Differences between assays with and without ejaculation

(A) Assays with ejaculation have fewer episodes of anogenital chemoinvestigation (KS-test P = 0.0098) and mounting (KS-test P = 0.020) than those without ejaculation, but no significant difference in intromission number (KS-test P = 0.68). (B) In assays with ejaculation, sniffing bouts are shorter (KS-test P = 0.029), but show no significant difference in mount (KS-test P = 0.21) or intromission bout duration (KS-test P = 0.27). (C) In assays with ejaculation, males begin sniffing with the same latency (KS-test P = 0.95) but are quicker to begin mounting (KS-test P = 0.049) and intromitting (KS-test P = 0.00018) than in those without ejaculation. (D) Mean time between sniffing bouts is shorter in assays without ejaculation (KS-test P = 0.025), but does not differ in mounting (KS-test P = 0.99) or intromission (KS-test P = 0.93).

Figure 4-4

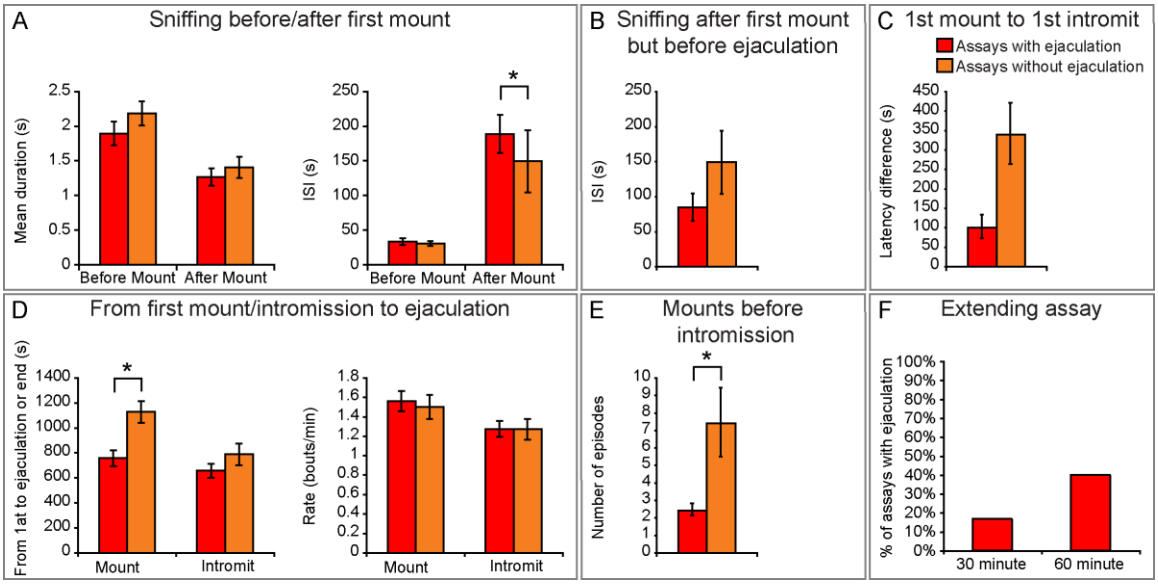

Figure 4-4 Controlling for mount latency.

Mean ± S.E.M. N = 31 assays with ejaculation, 35 without. (A) Differences in sniffing partly disappear when controlling for differences in mount latency. Mean duration of sniffing bouts before (t-test P = 0.25) or after (t-test P = 0.49) first mount is not significantly different between assays with and without ejaculation. Sniffing ISI is not different before the first mount (KS-test P = 0.98) but is still slightly shorter in non-ejaculators than in ejaculators after the first mount (KS-test P = 0.018). (B) Rate of sniffing is not significantly different between assays with and without ejaculation if ejaculation is considered the end of the assay (KS-test: P = 0.27). (C) The difference in time from the first mount to the first intromission is not significant (KS-test: P = 0.14). (D) If ejaculation is taken as the end of the assay, assays with ejaculation have much less time to mount (KS-test: P = $3.9 * 10^{-4}$), explaining the lower number of mounts (Figure 4-3A). Time available to intromit is the same between groups (t-test: P = 0.23). Rates of mounting and intromitting between the first mount or intromission and ejaculation or the end of the assay are almost identical (t-test: mount P = 0.72, intromit P = 0.98). (E) In assays with ejaculation, males proceed to intromission after fewer mounts (KS-test: P = 0.038). (F) An additional 30 minutes of assay time does not significantly increase the likelihood of ejaculation. (Animal's first assay only. Fisher's exact test: P = 0.22)

Figure 4-5

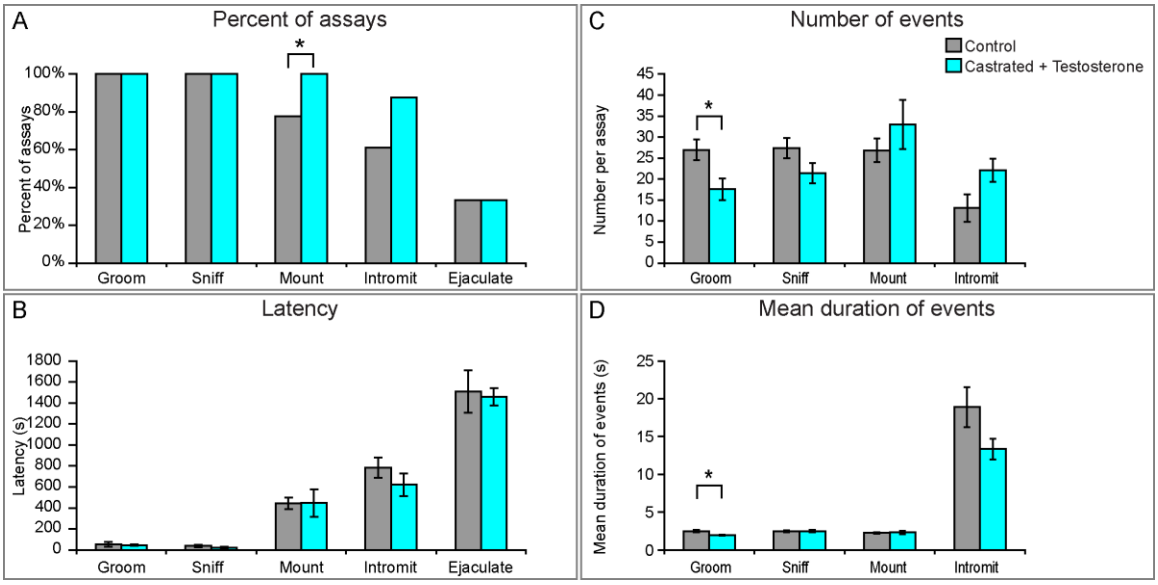

Figure 4-5 Homogenizing testosterone levels does not eliminate assay variability.

(A) Castrating males and treating with testosterone to decrease variability increases the likelihood of mounting (Fisher's exact test P = 0.027) but has no significant effect on the likelihood of intromission (Fisher's exact test P = 0.070) and leaves the probability of ejaculation unchanged. (B) Males castrated and treated with testosterone show no changes in latency to groom (KS-test P = 0.75), sniff (KS-test P = 0.35), mount (t-test P = 0.99), intromit (KS-test P = 0.33), or ejaculate (KS-test P = 0.45). (C) Males castrated and treated with testosterone show a decrease in number of grooming bouts (t-test P = 0.026) but no significant difference in sniffing (t-test P = 0.11), mounting (t-test P = 0.44), or intromitting (t-test P = 0.061). (D) Males castrated and treated with testosterone show a decrease in mean duration of grooming bouts (t-test P = 0.0085) but no significant difference in sniffing (KS-test P = 0.35), mounting (t-test P = 0.80), or intromitting (t-test P = 0.064).

Conclusions

In mating assays in which both external and internal inputs are controlled to the best of our ability, there is nonetheless a high degree of assay-to-assay variability in mating success, due only in part to stable differences between individuals. We find that a key

predictor of mating success is the latency to begin mounting, implying that the transition from sniffing to mounting represents a key moment of choice to begin an otherwise stereotyped pattern.

These results have already begun to inform the analysis of behavior in other experiments in our lab. Xu et al. identified sexually dimorphic genes that control specific parameters of individual sexually dimorphic behaviors. In particular, they found that males mutating *Sytl4* decorrelated intromission latency and likelihood of ejaculation without changing the overall latency to intromit or probability of ejaculation, demonstrating that the gene modulates the mating pattern we have described, a more subtle phenotype than would be revealed by looking at each behavior independently (Xu et al., 2012).

It remains unclear what determines the latency to begin mounting. The pattern of wild-type mating, in which there is intense anogenital chemoinvestigation before the first mount but much less once mounting has begun, suggests that the animal is gathering sensory cues which will be integrated with internal cues, such as the animal's hormonal state, which then leads to the choice to mate or not mate. In Chapter Two we demonstrated that chemosensory cues play an important role in the choice whether to begin the mating process, but not in the pattern of mating once begun. Positive chemosensory information, however, is not absolutely required for mating. Moreover, the presence or absence of MOE or VNO function, while affecting the probability of mating, did not affect the latency. That is, it affects the output of the choice, but not the timing. In contrast, in females, lack of MOE input not only does not decrease the likelihood of VNO- females mounting males, but actually decreases the latency (Chapter

Three).

What determines when that choice is made or carried out? In these wild-type assays, all female intruders were genetically identical and hormonally homogeneous. Nevertheless, their pheromonal profiles or proceptive behaviors may vary slightly. On the other hand, variability may primarily reflect the internal state of the males. Testosterone levels alone cannot account for these differences. Animals may differ in early life experiences such as past dominance. It remains to be determined how internal and external cues are integrated to control the choice and timing of mating.

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