UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Meaning of Mups: understanding the basis of activation in the vomeronasal organ

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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2010

The Thesis of Kathleen Marie Lloyd is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2010

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ACKNOWLDEGMENTS

I would like to acknowledge Professor Lisa Stowers for her guidance and help as my committee chair. I would like to thank Darren Logan for his mentorship in the lab. I would like to additionally thank Angeldeep Kaur for her assistance in the aggression assays. I would like to thank Pablo Chamero, Tobias Marton, and Angeldeep Kaur for their previous work in this field that set the foundation for my work, and for allowing me to use adaptations of their figures in this thesis. Finally, I would like to thank all of the members of the Stowers Lab for their input and help throughout my project.

Figure 7, in full, is adapted from work done by Angeldeep Kaur and Tobias Marton. This work is currently being prepared for submission.

Figure 8, in full, is adapted from work done by Angeldeep Kaur and Tobias Marton. This work is currently being prepared for submission.

ABSTRACT OF THE THESIS

The Meaning of Mups: understanding the basis of activation in the vomeronasal organ

by

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Master of Science in Biology

University of San Diego, California, 2010

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Animals use pheromones to communicate information such as social status and to elicit stereotypic behaviors such as intraspecies aggression, fear, reproduction, and suckling. The family of Major Urinary Proteins (Mups) in mice has been implicated as pheromones that promote individual recognition and male to male aggression. Mup variants 24 and 25 are sufficient to elicit stereotypic male to male aggression through

activation of the vomeronasal organ, part of the accessory olfactory system. The neural mechanisms by which these pheromones elicit this behavior however are largely unknown. Through the engineering of point mutations and Mup chimeras we found that the amino terminus domain of these proteins is required for specific neuron activation in the vomeronasal organ. Furthermore, a chimeric Mup 25, which activates a subset of sensory neurons stimulated by native Mup 25, fails to initiate Mup 25-mediated aggression in behavioral assays. Overall, our results provide a possible mechanism for Mup-receptor interactions as well as insight into the mode of information coding in the vomeronasal organ.

Introduction

Animal communication through chemosignals

In many mammalian species, pheromones and olfactory cues are essential for communicating information. These cues may be obtained from the environment or from other animals and function as either inter- or intra-specific cues. Pheromones and olfactory cues have been implicated in stereotypic behaviors such as intraspecies aggression, fear, reproduction, and suckling (Blass and Teicher, 1980; Boehm et al., 2005; Brechbuhl et al., 2008). These innate behaviors have been shown to be directed through pheromones in a contextually based manner. The neural circuitry by which these cues modulate behavior however is still largely unknown. Understanding the neuronal mechanisms behind these innate behaviors is a key factor in broadening our understanding of how the brain encodes and interprets external stimuli.

Interspecific communication: prey vs. predator response to chemosignals

Chemical communication between animals can occur between members of the same or different species. Interestingly, the molecules that mediate these interactions as well as the pathways utilized can be the same in both conspecific and heterospecific chemical communication (Papes et al., 2010). Elucidating the mechanisms of detection in the sensory system can therefore explain how an animal differentiates between predator and conspecific cues.

Interspecies communication can be defined as an exchange between members of different species through chemical cues. These semiochemicals can be excreted by either prey or predator. These may be pheromones that are excreted by a member of a

prey species in an intraspecific manner, while the predator species has evolved a detection method to this semiochemical for better hunting the prey. Alternatively, a prey species may evolve innate aversion or fear responses to the cues from a predator, enhancing the prey's ability to survive in a hostile environment (Apfelbach et al., 2005). Mice display avoidance behaviors when exposed to predator odorants, such as cat collars or red fox feces (Dell'Omo et al., 1994; Samuelsen and Meredith, 2009). Female mice have been found to decrease both litter size and foraging activity when exposed to predator cues. While the cues behind several of these behaviors have been discovered and characterized, the neural circuitry and mechanisms underlying this informational coding is not yet fully understood.

Intraspecific communication: cues for reproduction and social status

While the detection of interspecific cues may aid in the fitness of a particular species, this will do an animal no good if it cannot carry out social behaviors such as mate choice and reproduction. In higher mammals, this can be achieved through vocalization, however lower mammals are highly dependent on a chemical mode of communication (Zarzo, 2007). This consists of either odorants which can be associated with specific situations and acted upon accordingly, or pheromones which instigate an innate response.

Intraspecies communication is the dialogue between members of the same species through specific signals or cues. These cues may mediate behaviors such as reproduction (Boehm et al., 2005), territorial scent marking (Nevison et al., 2003), pup suckling (Schaal et al., 2003; Teicher et al., 1980), oestrus induction (Bronson and

Whitten, 1968; Mucignat-Caretta et al., 1995), or as elucidated in this study, male to male aggression (Novotny et al., 1985). Males will aggress against another male when paired in one cage, but not against a castrated male. Addition of male urine to the back of a castrate mouse will initiate this aggression behavior (Chamero et al., 2007). This is a contextually based cue, as male urine applied to pups or food will not instigate stereotyped aggression (Mucignat-Caretta et al., 2004). Dominant mice will also display a countermarking behavior in response to sensing a more submissive males' urine in their cage. In females, pregnancy may be aborted when a female is exposed to unfamiliar male scents, known as the Bruce effect (Bruce, 1959). Each of these behaviors has been found to be instigated by conspecific cues. To better understand how these behaviors are processed in the brain, we must first investigate how these signals are detected and distinguished by the olfactory sensory system.

Transduction and informational coding of pheromones and odorants

How are pheromones and odorants detected by sensory systems and encoded in the brain for the transduction of chemical communication? Both pheromones and odorants may activate dual pathways in the olfactory sensory system. This system consists of two sensory organs called the main olfactory epithelium, or MOE, and the vomeronasal organ, or VNO. The MOE is located in the posterior dorsal region of the nasal cavity, while the VNO resides in a cartilage casing at the base of the nasal septum (Halpern, 1987; Lin et al., 2004).

Traditionally, the main olfactory system had been implicated in the sensing of odors, while the accessory olfactory system has been implicated in pheromone

sensing. Primary signal transduction in the VNO and the MOE has been found to be molecularly distinct (Dulac and Torello, 2003). Both the VNO and the MOE express G-protein coupled receptors (GPCR) in their respective sensory neurons, but they are from distinct protein sub-families (Kaupp, 2010).

Olfactory receptors (ORs) expressed in the sensory neurons of the MOE are capable of detecting a wide range of small molecule odorants (Buck, 2004). The activation of ORs leads to the opening of CNGA2 channels. These neurons synapse onto the main olfactory bulb (MOB), which then projects to mitral cells that synapse on to the cortex and the olfactory amygdala (Dulac and Torello, 2003) (Figure 1a).

In the VNO, there are two receptor classes that are expressed, the V1Rs and the V2Rs (Keverne, 1999; Ryba and Tirindelli, 1997). The activation of phospholipase C leads to the opening of TrpC2 channels in both vomeronasal neurons (VNs). Activated VNs synapse to the accessory olfactory bulb (AOB), which then projects neurons directly to the vomeronasal amygdala and hypothalamus (Dulac and Torello, 2003)(Figure 1b). These molecular and spatial organizations of the main and accessory olfactory systems indicate that they may perform differentiated roles in the detection of odorants and pheromones.

However, while these two systems do in fact have differential roles, they also have a certain amount of overlap (Meredith, 1998). Certain behaviors known to be modulated by pheromones are not eliminated when the VNO is ablated. Analysis of *Trpc2*$^{-/-}$ mice has shown that the ablation of VNO function was not sufficient to eliminate either the mating or suckling response in mice, behaviors previously thought

to be modulated by pheromones (Leypold et al., 2002). Conversely, in experiments with *Cnga2*$^{-/Y}$ mice and MOE glomeruli ablations it was found that aggression, mating and innate aversion was attenuated, suggesting a broad modulating role of the MOE on VNO function (Kobayakawa et al., 2007; Mandiyan et al., 2005). Overall, this previous work shows that while these traditional roles for the VNO and MOE hold true in some cases, the precise mechanisms behind pheromone and odorant differentiation are as yet unknown. For further research to be done on these non classical modes of pheromone and odorant detection, it will first be beneficial to fully elucidate what mechanisms are underlying the classical circuits found in these two systems. Understanding the mechanisms behind pheromone transduction in the VNO can help us understand the molecular basis behind possible differentiation in the function of these organs.

Ligand detection is organized by spatial organization of the VNO

While the VNO is known to have a role in the detection of pheromones, the varied neuronal makeup of this subsystem has made it difficult to elucidate the receptors responsible for innate behaviors (Bush and Hall, 2008). This is compounded by the fact that different pheromones have diverse chemical and structural characteristics. Vomeronasal neurons respond to female and male urine differentially in a manner that indicates quantitative interaction of a receptor with a ligand (Holy et al., 2000). Thus these receptors found to be expressed differentially in the VNO may have evolved to associate with specific ligands, creating innately wired pathways to mediate stereotypic behaviors.

The VNO is capable of detecting both small volatiles and nonvolatile peptides (Dulac and Axel, 1995; Wysocki et al., 1980). How does the VNO differentiate between large and small molecule pheromones? This is the first step in elucidating molecular mechanisms behind pheromone coding.

In the main olfactory system, dispersed sets of neurons are activated in response to odorants. These cells converge onto molecularly distinct glomeruli. The VNO however has a heterologous organization of neurons onto the glomeruli in the AOB (Del Punta et al., 2002). Additionally, there is a spatial organization of molecularly distinct neurons in the VNO. The VNs are spatially separated in the VNO, with V1Rs expressed apically and V2Rs expressed basally. Additionally, the Gαi2 proteins have been found to complex specifically with the V1Rs, while Gαo proteins have been found to complex with V2Rs (Krieger et al., 1999)(Figure 1c). The V1Rs and V2Rs in the VNO additionally have distinct electrical responses to stimuli, further dividing the apical from the basal VNO (Fieni et al., 2003). Finally, the V1R and V2R receptors project to the apical and basal regions of the AOB correspondingly, establishing subdivisions of glomerular projection (Jia et al., 1997) (Figure 1c).

The capacity of the VNO to detect pheromones can be defined by the large number of family members found in both the V1R and V2R classes of receptors. The V1R class consists of 139 receptors which can be organized into 12 distinct families (Rodriguez et al., 2002). A subset of stereotyped behaviors controlled through the VNO was found to be dependent on intact V1R receptors including male sexual

behavior and maternal aggression, stimulated by small molecules (Del Punta et al., 2002).

The V2R class similarly consists of 121 receptors, organized into 12 families (Yang et al., 2005) (Figure 2a). As an additional level of complexity, instead of following the "one neuron one receptor" rule of the main olfactory system, the basal VNO sensory neurons express multiple receptors per neuron (Fleischer et al., 2009; McClintock, 2010). This makeup is currently thought to consist of one of the 6 "C" family receptors expressed in every basal neuron, with an additional V2R from families A or B or co-expressed in these same cells (Martini et al., 2001) (Figure 2b). The function behind the heterologous co-receptors expressed in these neurons is unknown.

The functional relevance of the V2R-expressing VNs has begun to be elucidated. Pheromone proteins expressed in male mouse urine are sufficient to stimulate innate aggression in males through activation of the VNO, specifically through neurons expressing Gαo and thus V2R receptors (Chamero et al., 2007). This stereotyped behavior provides an excellent platform to further understand the expression pattern of the VN receptors.

Pheromones can be large or small molecules

Pheromones were initially detected when the compound N-acetyl teramine was discovered to be responsible for copulation in the silk moth *bombyx mori* in 1959 (Butenandt et al., 1959). Since then, the question of pheromones used in the mammalian world has become increasingly prevalent. In 1959, Karlson and Luscher

defined pheromones as “substances which are secreted to the outside by an individual species, in which they release a specific reaction, for example, a definite behavior or a developmental process” (Karlson and Luscher, 1959).

Since the VNO lumen is fluid filled and mostly inaccessible to volatile molecules, pheromones were previously thought to be nonvolatile semiochemicals (O'Connell and Meredith, 1984; Stowers and Marton, 2005). However, several volatile compounds have been identified that cause stereotyped behaviors in mice. These cues have been shown to elicit behaviors such as maternal aggression, puberty acceleration and delay, and female attraction (Novotny et al., 1985; Novotny et al., 1986; Novotny et al., 1999). Additionally, an innate response to the volatile chemical MTMT was found to be detected through the MOE, a nontraditional mode of detection for innate behaviors (Lin et al., 2005). Although traditionally, volatile molecules were classified as odorants while nonvolatile semiochemicals were thought to be exclusively pheromones, these compounds show that a volatile compound can indeed cause a stereotyped behavior in a pheromone-like manner.

As volatiles cannot easily access the mucous-filled lumen of the VNO, one mechanism of action for these potential volatile pheromones to be transported can be found in the mouse Major Urinary Proteins, or Mups. These are a family of genes that belong to the lipocalin superfamily. Lipocalins consist of eight anti-parallel beta sheets that form a hydrophobic binding pocket in the middle, which binds various small hydrophobic molecules in the calyx groove (Flower, 1996; Ganfornina et al., 2000; Timm et al., 2001) (Figure 3). The small hydrophobic molecules that bind this region

of the Mup include all six identified male specific pheromones (Beynon and Hurst, 2003). These molecules were shown to bind to different Mups with different affinities, showing that Mup proteins can form complexes with a number of these possible ligands, or carry no ligand at all (Bacchini et al., 1992). Thus, this evidence supports the role of Mups as carrier proteins to deliver these volatile pheromones to the VNO.

In addition to volatile pheromones, proteins have been implicated in pheromone response. The protein aphrodisin is expressed and excreted in vaginal fluid in female hamsters, and is sufficient to elicit copulation behaviors in males (Singer et al., 1986). This protein, once analyzed for primary structure was found to be similar to Mups in that it was a member of the lipocalin superfamily, indicating a broad role for these proteins in modulation of innate behaviors (Henzel et al., 1988). In addition to this, the MHC class I peptides were shown to act as individuality signals in the context of mate recognition during pregnancy block (Leinders-Zufall et al., 2004). Finally, a peptide excreted from the exorbital lacrimal gland called exocrine gland-secreting peptide 1 (ESP-1), was found to be secreted in the eyes of males and specifically activate female VNOs through direct contact (Kimoto et al., 2005).

While these examples show precedent for pheromonal activity of a peptide, Mups are unique for their role in volatile pheromone transport. Since the protein aphrodisin shares characteristics with Mup proteins, there is the added possibility of endogenous pheromone activity in Mups. Mups could activate the VNO individually or through creating complexes with their volatile pheromone ligands.

The Major Urinary Proteins of Mice are diverse, highly homologous genes

Mups have been previously implicated in volatile pheromone transport; however a large expansion in the mouse genome indicates a more diverse functionality than acting as a simple carrier molecule (Logan et al., 2008). Interestingly, although there is a large genomic cluster of Mups, they have an extremely high sequence homology at both the protein and nucleotide level (Logan et al., 2008).

Mups were initially found to be synthesized in the liver of male mice and excreted as the most prominent protein in the urine at concentrations of 10 to 30 mg/ml (Finlayson et al., 1965; Rumke and Thung, 1964). Indeed, they are the most abundant transcript found in the liver, consisting of 5% of all liver cDNA and present at 30,000 copies per cell, indicating that they serve an important function to warrant so much energy in expression (Hastie et al., 1979). Mups were later found to also be excreted by the submaxillary, lachrymal and mamillary glands (Shaw et al., 1983).

Mup transcription has been shown to be dependent on several hormones. Adult male mouse Mups are not expressed in either females or castrates, however this expression profile has been rescued by testosterone (Szoka and Paigen, 1978). Mice can therefore easily be created as “Mup nulls” through castration when studying Mup-mediated male to male interactions. Additionally, GH deficiency was found to deplete the expression of Mups by 150 fold, and testosterone treatment was found to be insufficient for Mup rescue (al-Shawi et al., 1992; Knopf et al., 1983). Therefore, both hormones have a distinct yet complementary effect on Mup production in mice.

There are at least 21 Mup genes present on chromosome 4 (Clark et al., 1984; Logan et al., 2008). Mouse Mups exist in two distinct classes, the class As and class Bs (Logan et al., 2008) (Figure 4a). Mups are expressed in different profiles of six to eight Mups across strains in laboratory mice and individuals in wild mice, creating an individuality "barcode" (Hurst et al., 2001; Robertson et al., 1997). The male C57Bl/6J mice used in this study all express the same 5 Mups from their genome (Figure 4b). The class A Mups have a higher level of divergence, while the class Bs have been found to be highly homologous, containing only 8 residue polymorphisms between the 15 C57Bl/6J class B Mups (Figure 5).

Mups have been shown to be an important factor of individual recognition and territory ownership in male mice (Beynon and Hurst, 2003; Darwish Marie et al., 2001; Nevison et al., 2003). Male mice will not countermark a submissive mouse's urine if the stimulus is covered by a nitrocellulose membrane allowing volatiles through, implicating Mups in this behavior (Hurst et al., 2001). Finally, females discriminate during mate choice to favor males with increased heterozygosity of Mups (Thom et al., 2008).

In addition to this, stereotypic male to male aggression has been shown to be mediated by both purified and recombinant (therefore lacking any volatile molecules) Mups (Chamero et al., 2007). This behavior is particularly helpful when studying Mup-mediated behaviors, as creation of Mup "nulls" through castration also ablates any innate aggression an intact male has towards the castrate. However, if a castrate has Mup stimulus applied to its back (Figure 6a), an intact male will respond with

stereotyped aggression behaviors (Figure 6b). Both the Mup-containing fraction of male urine and recombinantly expressed male Mups have been shown to be sufficient for this behavior (Chamero et al., 2007). Beyond the pool of Mups, when tested individually it was found that Mups 24 and 25 were individually sufficient for male to male aggression (Figure 7).

Highly homologous Mups activate differential, specific subsets of neurons

Mups are sufficient for inter-male aggression without binding volatile aggression pheromones (Chamero et al., 2007). As Mups are small (~20 kDa), inherently stable molecules, we have designed recombinant Mups that retain their native neuronal activation profiles with a Maltose Binding Protein (MBP) tag to allow for protein purification. This has allowed for experiments with specific Mups absent of any bound molecules. Using these recombinant proteins, it was found that Mups 24 and 25 alone are individually capable of eliciting stereotypic aggression (Marton et al, data not published, Figure 7). How are Mups 24 and 25 activating VNO neurons in a manner sufficient for aggression? As these are the only Mups that have been identified in our lab to induce an easily quantifiable stereotyped behavior, they are also ideal to study for the question of detection mechanisms in the VNO.

The vomeronasal activation seen from Mups 24 and 25 will help to elucidate the neural mechanisms driving this innate behavior. The vomeronasal organ is differentially activated by different Mup ligands, despite the high homology of Mups (Papes et al., 2010; Marton et al, data not published) (Figure 8). Each of the five Mups expressed in C57Bl/6J urine are sufficient to activate a unique subset of neurons

specific to each recombinant Mup stimuli, called specialist neurons (Figure 8a, i-iv). Additionally, there are four subsets of neurons activated by various sequential combinations of Mups, called generalist neurons (Figure 8b, v-viii).

The divergences in Mup sequence and structure lead to individual coding in the VNO, possibly through different receptor-ligand pairs. How might this interaction be occurring? Given the heterologous expression of receptors in the basal VNO, the most similar system known are the sweet taste receptors (Konstantinidis, 2009). The regions of sweet proteins that drive binding and neuron activation are likewise unknown, although there is some indication that there are non-traditional mechanisms of protein-to-receptor interaction (Temussi, 2002). There are no immediately apparent regions when examining polymorphisms between Mup proteins where a traditional binding pocket might bind. Through point mutations in the highly homologous class B Mups and construction of Mup chimeras consisting of the aggression-promoting class A Mup 25 and the class B Mup 8, we can investigate the regions of the Mup protein involved in information detection in the VNO. Elucidating the basis of neuronal coding of Mups will allow us to further understand how sensory neurons in the VNO are activated in a specific manner by such a highly homologous class of ligands, and grant further insight into the processes that allow specific Mups to instigate a specialized pheromone response.

Materials and Methods

Bioinformatics

C57Bl/6J mouse Mup sequences were acquired from the annotated genome for sequence analysis. The accession numbers for each Mup can be found in Table 1. V2R sequence information was acquired from the annotated genome, compiled by Barbara Trask (Lane et al., 2004). Phylogenetic trees were created using the MEGA 4.1 program, via a Neighbor Joining method and using a Kimura 2-parameter model. Sequences alignments were created using Clustal W, and formatted with GeneDoc.

Recombinant Mup and Chimera Construct Design

RNA was extracted from the liver of a male C57BL/6J mouse, using an RNeasy extraction kit (Qiagen, Valencia, USA) and reverse transcribed using an oligo-dT primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, USA). Mups were cloned from the synthesized cDNA using primers designed with the restriction sites *BamHI* and *HindIII* introduced in the forward and reverse primers respectively (Table 2). Mups were subcloned into the pCRII-TOPO vector (Invitrogen), and sequenced against the annotated genome. Mups 3, 8, 17, 24, and 25 were then cloned into the pMal-c2x vector (New England Biolabs).

The Class B Mups 4, 7, and 13 were mutagenized from Mup 17 inserted in the pMal-c2X vector with primers annotated in Table 3. The QuikChange II Mutagenesis Kit was used for mutagenesis reactions (Agilent Technologies). Plasmids containing mutagenized Mups were sequenced to ensure that the mutation had occurred.

Chimeras were designed using Mups 8 and 25. Residues 1 to 66 of Mup 25 was merged to residues 67 to 180 of Mup 8, and designated as N25. The second chimera was designed with the amino terminus of Mup 8 from residues 1 to 66 and the carboxyl terminus of Mup 25 from residues 67 to 180, and the chimera was labeled as N8. Chimeras were designed with a *BamHI* restriction site at the 5' end of the sequence, and a *HindIII* restriction site at the 3' end. Chimeras were synthesized by Genscript, and ligated into the expression plasmid pMal-c2X (New England Biolabs) with Maltose Binding Protein at the carboxyl terminus for purification of expressed protein.

Protein Preparation

Recombinant Mups in pMal-c2X expression plasmids were transformed into DH5α bacterial cells (Invitrogen), and a 10 ml culture was grown up overnight in LB medium. Bacterial culture was then inoculated into 1 L of LB medium, 2 g Glucose and 100 ug/ml ampicillin, and grown until the OD reached 0.5 at 590 nm. Cells were then induced with 0.3 mM IPTG and bacteria were further incubated for two and a half hours with shaking at 37 °C. Cells were spun down for twenty minutes at 4 °C at 4,000xg.

Cells were resuspended in 25 ml Column buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 200 mM NaCl made in ddH_2O) and one dissolved protease inhibitor cocktail pill (Roche). Cells were lysed for thirty minutes on ice with 1 mg/ml chicken egg white lysozyme, or 25 mg in 25ml of suspension (Sigma). Cells were sonicated by

probe for two minutes at 28% for 3 second on/off cycles and spun down for thirty minutes at 4 °C at 9,000xg.

Supernatant was collected and recombinant proteins were captured using 4 mL of 50% amylose resin slurry (New England Biolabs) overnight at 4 °C. Protein was eluted with 2 mL of 25 mM Maltose in Tris-HCL buffer. Proteins were stored at 4 °C and used within two weeks.

Calcium Imaging

Primary vomeronasal neurons (VNs) were prepared from 8- to 10-week old male C57Bl/6J mice by dissection in ice cold 1X PBS. VNs were first mechanically dissociated in ice cold papain solution (0.0022 U papain, 5 mM cystein, and 2.5 mM EDTA in 1X PBS) followed by chemical dissociation with papain solution at 37° C with shaking for twenty minutes. 5 ul of DNase was then added to the solution to prevent cells from clumping, followed by addition of 10 ml DMEM. Cells were pelleted at 1.1 rcf for five minutes and the supernatant was removed. Cells were then resuspended in 80 ul DMEM and pipetted onto coverslips coated with concanavalin A (Sigma Aldrich). Dissociated VNs were perfused with stimuli at 37 °C, and intracellular calcium was monitored using a 1:100 dilution of fura-2/AM (Molecular Probes) in calcium imaging buffer (5 mL of HEPES in 500 mL of 1X HBSS buffer) by a Zeiss Axiovert 200M inverted microscope.

Urine was collected from 8- to 12-week-old C57Bl/6J males and used as a positive control in experiments. Recombinant Mup proteins were diluted in calcium imaging buffer at a concentration of 50 ug/ml. Stimuli was perfused for 60 seconds

with 120 second buffer-only intervals. Traces of neurons were normalized to the y value of the first data point, creating a baseline normalized to 1. Transients were only counted as valid responses if the trace began before the 60 second window of stimulus had ended, and if the transient responded at least 1.5 times above the base level (1 Unit or more of increase). The base level of the neuron was calculated as the average of the ten data points before the beginning of each stimulus.

Each population of neurons was compared to a negative control experiment with Maltose Binding Protein (MBP) in calcium imaging buffer as a stimulus. Responding neurons were only counted if they responded to a 60 second perfusion of urine as a positive control. Each population of neurons from experiments was compared to the equivalent number of responses in the negative control using a Mann-Whitney U statistical test. Populations were only considered significant if the p value was less than 0.05.

Aggression Assays

Eight to twelve-week old C57Bl/6J male mice were isolated for at least one week before conducting behavioral experiments. Males were castrated at three weeks of age, and used in assays after at least eight weeks of age. Castrates were painted with 40ul of 5mg/ml stimuli onto their backs and paired with an intact male. Pairings lasted for ten minutes and were assayed for the total duration of aggressive behavior in that time period.

Tests took place in the home cages of isolated mice. Tests were videotaped and analyzed at quarter speed using Observer software (Noldus Technology) to measure

aggression parameters including tail rattling, biting, chasing, cornering, tumbling and kicking. Total duration was defined as the total duration of aggressive contact behavior consisting of kicking, biting, wrestling or tumbling.

One round of urine as a positive control and no-urine as a negative control was performed with each resident mouse before and after sample testing. Results of the experimental stimuli were compared to both the positive and negative controls using a Students Paired two tailed T-test. Results were only considered statistically significant if the p value was less than 0.05.

Results

Alignment of Class A and Class B Mups

How are individual Mups sufficient to activate specific populations of vomeronasal neurons? One possibility is that specific regions of the Mup protein are essential for receptor to ligand interaction. To determine the specific domain of the Mup protein that is sufficient to dictate a specific neural response, we first looked at an alignment of all of the Mups found in the C57Bl/6J genome (Figure 5). The C57Bl/J6 mouse Mup alignments were divided by the class As and the class Bs. Class A Mups are more divergent, containing 66 total divergent residues across the entire class. The class B Mups possess significantly fewer polymorphic residues when examining the amino acid sequence, with a total of 8 polymorphic residues across the 16 members of this class (Figure 5).

Interestingly, even with such a low amount of divergence, certain of these Mups can differentially activate vomeronasal neuron (VN) populations. For the rest of this study, Mup-responsive VNs will be referred to in two separate classes: generalists and specialists. The five Mups in C57Bl/6J urine are labeled 3, 8, 17, 24 and 25. Four sets of generalist neurons will respond to either class A Mups, class B Mups, to every Mup but Mup 24, or to any class of Mups indiscriminately (Figure 8b,v-viii). These are referred to as 24/25 generalists (Figure 8b,v), class B generalists (Figure 8b,vi), No 24 generalists (Figure 8b,vii) and Mup generalists (Figure 8b,viii) respectively (Marton et al, data not published). In addition to generalist neurons, each of the Mups expressed in C57Bl/6J male urine is sufficient to activate specialist neurons in

C57Bl/6J VNs. These are neurons that will respond exclusively to a specific Mup, even when stimulated with other possible stimuli (Marton et al, data not published, Figure 8a,i-iv).

The class B Mups are a good platform to elucidate the domain of Mups interacting with VN receptors, as most class B Mups only differ by one or two amino acids in their primary sequence (Figure 9a). Mups 3, 8 and 17 have previously been cloned and used in calcium imaging experiments, and each have been shown sufficient to activate a specific subpopulation of neurons (Marton et al, data not published, Figure 8a,i-iv). Of these three Mups, Mup 8 contains a E50K substitution, while Mup 3 possesses a F56V and a Q140K substitution (Figure 9a). Of these three residues, two reside in the amino terminus half of the Mup. When examining a crystal structure of the Mup protein, it is found that the amino and carboxyl halves of the primary protein sequence form different faces of the Mup (Figure 9b). The single residue polymorphism of Mup 8 resides on the amino terminus of the Mup, indicating that this face may be important in receptor-ligand binding and specific ligand recognition.

Residues implicated in specialist coding through activation profiles of class B Mups

To address the question of which face of Mups may be involved in specialist coding, we looked at additional activation profiles of four C57Bl/6J Mups – Mup 17, Mup 13, Mup 7 and Mup 4. These were chosen because each possesses one, two or neither of two residue polymorphisms that reside in the amino and the carboxyl terminus respectively (Figure 10a). Mup 4 and Mup 13 contain an E13Q substitution, while Mup 7 and Mup 13 contain a Q140K substitution (Figure 10b). Mup 17

possesses neither of these substitutions, and has previously been shown sufficient to activate a specialist population of neurons (Figure 8a,i-iv). With these Mups, we can investigate whether one or both of these substitutions are sufficient to elicit a specific response from VNs. To do this, we used dissociated primary neurons from the VNO (see Materials and Methods). Previous work has shown that when these neurons are loaded with fura-2, they will respond transiently to relevant stimuli (Chamero et al., 2007). Calcium imaging of these transient responses can be recorded at the resolution of single cells to determine which ligands are sufficient to propagate a signal in specific neurons.

Each of the Mup stimuli was perfused over primary VNs consecutively with perfusions of stimulus-free buffer to clear the stage of stimuli. Data was collected and represented as the percent of individual neurons that responded to different subsets of consecutively pulsed Mups. Populations of neurons were counted only if they were significantly above the percentage of neurons responding to the same number of Maltose Binding Protein (MBP) control perfusions, referred to from here on as “noise”.

A specific population of specialist VNs found to be significantly above noise responded to separate pulses of Mups 4 and 13 at a rate of 0.465% of total cells (n=39, $p<0.0001$) (Figure 11a, 12). An additional significant population of specialist neurons responded to pulses of Mups 7 and 17 at a rate of 0.427% ($p<0.0001$) (Figure 11b, 12), and a final significant population of generalist neurons responded to all four Mup pulses at a rate of 0.876% ($p<0.0001$) (Figure 11c, 12).

Mups 4 and 13 both contained the K13Q substitution (Figure 10). Mup 13 also possessed the additional E140K substitution, but this was not sufficient to activate a unique population of neurons significantly different from noise. All neuron response to Mup 13 was contained in Mup 4 responsive neurons, and vice versa (Figure 12). Mups 7 and 17 consecutively activated a subset of neurons (Figure 11b), but neither of these Mups activated neurons that could differentiate between them (Figure 12). Mup 7 possessed the E140K substitution, which resides on the carboxyl 'face' of the Mup (Figure 10). Taken together, these response profiles show that the single substitution on the amino face of the Mups is sufficient to encode a specific neuronal response, but the single substitution on the carboxyl terminus is not.

Design of a Mup 8 and Mup 25 Chimera

Although the class B Mup experiments support the hypothesis of specificity coding through the amino terminus of Mups, the aggression promoting pheromones of the class A family provide a more rigorous test of this theory. The class A Mups lack this extremely high level of sequence homology, so single residue mutations are not practical for the class As. However, polymorphic residues of class A Mups are also found in the amino terminus of the primary sequence (Figure 13a), indicating that differentiated signaling may occur through this face.

We designed a chimera consisting of the amino terminus of one Mup and the carboxyl terminus of another Mup. The chimera was designed from class B Mup 8 and class A Mup 25. We designed a chimera with Mup 25, which along with specialist neuron activation also causes a specific stereotyped behavior (Figure 7, 8a, iv). Mup 8

was used for the second half of the chimera as it is one of the most divergent from Mup 25 and also is a member of the class B family, which has been shown to possess the ability to activate specialist neuron responses in the amino terminus (Figure 8a,ii).

Two chimeras were designed: one with residues 1 through 66 of Mup 25 followed by residues 67 through 162 of Mup 8, and one with residues 1 through 66 of Mup 8 followed by residues 67 through 162 of Mup 25 (Figure 13b). They were labeled N25 and N8, respectively. These amino terminus domains (NTDs) each included the first three beta sheets of the protein beta barrel (Figure 13a). The N8 chimera contains the single amino acid divergence found in Mup 8 at position 50, and so would be expected to activate 8 specialist neurons. Since both Mups 8 and 4 have been shown to activate a specialist neurons, we tested the extent to which the N25 chimera will support this finding, and activate Mup 25 responsive neurons specifically.

Residues 1 to 66 of Mups 25 are sufficient for a specific neuron response

The N25 chimera was examined for the level of activation overlap when compared to the native Mups it was designed from. The N25 chimera was perfused onto a coverslip with dissociated VNs, followed by Mup 8 and Mup 25 in an intentionally random order. There were 4959 cells imaged (n=21). Four populations of neurons were significantly above noise: Mup 25 specialists that responded to N25 (Figure 14a), Mup 25 specialists that did not respond to 25 (Figure 14b), Mup 8 specialists (Figure 14c) and Mup generalists responding to all stimuli (Figure 14d).

The majority of Mup 25 specialist and Mup generalist responses were accounted for by the N25 chimera, while the opposite was true for Mup 8 specialists, implicating the NTD of Mup 25 in a specialist neuron response (Figure 15). Neurons responding to Mup 25 and the N25 chimera were found to be significantly above noise, responding at a rate of 0.783% ($n=21$, $p<0.0001$) (Figure 14a, 16). Mup 25 activated a slightly significant population of neurons consisting of 0.349% ($p=0.0193$) of total cells (Figure 14b, 16). In contrast, 0.645% of these neurons responded to Mup 8 specifically which is significantly above the level of noise ($n=21$, $p<0.0001$) (Figure 14c, 16). Neurons that responded to both Mup 8 and N25, however were not significantly greater in number than those that responded to two pulses of MBP ($n=21$ $p=0.1074$) (Figure 16). Neurons responding to the N25 chimera specifically were not significant at 0.256% ($p=0.0536$) (Figure 16). Finally, a population of generalist neurons was activated by all four stimuli at 1.209% of urine all responsive cells ($n=21$, $p<0.0001$) (Figure 14d, 16). This data implicates the NTD of Mup 25 in specialist neuron activation. Since there is no overlap of the N25 chimera with the Mup 8 responsive neurons, this indicates that the C terminus domain (CTD) of the chimera is not sufficient for a specialist neuron response.

There are two populations of neurons from this experiment that bear further investigation – the Mup 25 responsive pool and the N25 responsive pool. These populations seemed to be higher than the usual level of noise, although the N25 only population was not significant (Figure 16). To further investigate the robustness of these populations, a double pulse experiment was conducted to account for a possible

drop off of neuronal responses when the same stimulus is pulsed twice. When the same stimulus is pulsed twice followed by a positive control, 34.78% of responding neurons did not respond to the second stimulus (Figure 17). The neurons responded to the same stimuli twice at a rate of 0.634%, while the percent of neurons responding to the first pulse only responded at a rate of 0.338%. Neurons that responded to the second pulse of identical stimulus were not significantly above noise ($p=0.1645$, $n=21$). These values are not statistically significant from the response rate of neurons responding to Mup 25 and the N25 chimera and neurons responding specifically to the N25 chimera, respectively (Table 4). These results confirm that the N25 responsive pool is *not* a significant population.

Residues 1 to 66 of Mups 8 are sufficient for a specific neuron response

The NTD of Mup 25 has been shown to activate a majority of the specialist response. Is the NTD of Mup 8 also sufficient for specialist behavior? The NTD of Mup 8 includes the polymorphic residue at position 50, delineating it from Mup 17, so there should be overlap with Mup 8 as opposed to Mup 17 specialist responses.

The N8 Chimera was assayed for specificity coding by stimulating vomeronasal neurons in addition to Mup 8 and Mup 25. There were 5965 neurons imaged ($n=27$). Three populations of neurons were seen at a rate significantly above noise: neurons responding to both Mup 8 and N8 (Figure 18a), specialist neurons responding to Mup 25 (Figure 18b), and generalist neurons responding to all stimuli (Figure 18c). The majority of Mup 8 specialist and Mup generalist responses were accounted for by the N8 chimera, while the opposite was true for Mup 25 specialists

(Figure 19). This data shows that the NTD of Mup8 in the N8 chimera is significant for a Mup 8 specialist response.

Interestingly, 0.747% of neurons responded to both Mup 8 and the N8 Chimera (42 responsive cells, $p<0.0001$) (Figure 18a, 21). Of all VNs imaged, only 0.166% that responded to Mup 8 did not respond to N8, which is not significantly above the level of noise (10 responsive cells, $p=0.3567$) (Figure 20). There was a significant response of neurons specifically to Mup 25 at 0.525% (34 responsive cells, $p=0.0001$) (Figure 18b, 21), while neurons responding to both Mup 25 and the N8 chimera did not rise significantly above noise at 0.0958% (5 responsive cells, $p=0.1902$) (Figure 20). Neurons responding to the N8 chimera alone were not significantly above the level of noise (11 responsive cells, $p=0.4112$) (Figure 20).

The N8 chimera accounts for all significant Mup 8 specialist neuron activity in the vomeronasal organ. There is no additional pool of Mup 8 specialists that fail to also respond to the N8 chimera. The N8 chimera is not sufficient for a Mup 25 specialist response however, indicating that the CTD of Mup25 lacks specialist activation.

<u>The N25 chimera is not sufficient for the aggression-promoting behavior of Mup 25</u>

The N25 chimera has been shown to activate Mup 25-specific neuron populations, while lacking the potential to activate Mup 8-specific neuronal populations and maintaining activation of generalist neurons. Given these neuron response profiles, the N25 chimera successfully mimics the specific activation of Mup

25. The next step is to determine if this chimera is sufficient for the stereotypic aggression behavior seen in males.

Castrate males were paired with intact male mice for the assay, and the respective stimuli was applied to the castrates' back. Mice were assayed against whole urine (n=25), blank castrates (n=17), Mup 25 (n=6), and the N25 Chimera (n=21). While Mup 25 initiated a significant level of aggression in mice at a duration of 13.98 seconds (p=0.00294) (Figure 21), the aggression observed from N25 stimuli was not statistically significant from the negative control (p=0.1919) (Figure 21). Thus the N25 chimera is not sufficient for the behavioral function of Mup 25.

The N25 chimera does not activate the 24/25 population of neurons in the VNO

The loss of aggression found in the N25 Mup could be from either a gain or loss of function in VNO activation. Since there is no significant population of neurons responding to N25 exclusively, the latter possibility is more likely. Although the N25 chimera overlaps with the population of Mup 25 specialist neurons, it may not overlap with the 24/25 responsive neuron population seen previously (Marton, data not published, Figure 8b,iii). This lack of neuronal activation could lead to the deficit seen in behavioral responses. To investigate this possibility, we conducted a calcium imaging experiment that stimulated VNO neurons with Mup 8, Mup 25, the N25 chimera and Mup 24. This will indicate if the N25 chimera activation overlaps with the Mup 24-Mup 25 responsive neurons, exclusively with the Mup 25-responders, or a novel sub-population of neurons.

There were 6219 total cells imaged in this experiment (n=19). Five populations of neurons were significantly above noise: Mup 8 specialists (Figure 22a), Mup 25 specialists that responded to N25 (Figure 22b), Mup 24/25 generalists that did not respond to N25 (Figure 22c), Mup 24/25 generalists that did respond to N25 (Figure 22d), and Mup generalists responding to all stimuli (Figure 22e).

The majority of Mup 25 specialists, No 24 generalists and Mup generalists also responded to the N25 chimera, however the chimera did *not* account for the majority of the Mup 24/25 generalists (Figure 23). There was a significant response of neurons specifically to Mup 8 at a rate of 0.763% of total cells, significantly above the level of noise (42 responsive cells, $p<0.0001$) (Figure 22a, 24). Neurons responding to Mup 8 and N25 however were not significantly above noise at a rate of 0.167% (10 responsive cells, $p=0.0673$) (Figure 24). This supports the previous N25 calcium imaging experiment showing that the CTD of Mup 8 is not sufficient for a specialist neuron response.

There was a significant population of neurons responding to both Mup 25 and N25 at 0.794% (38 responsive cells, $p<0.0001$) (Figure 22b, 24). A significant population responded to Mup 24 and Mup 25 at 0.519% (24 responsive cells, $p<0.0001$) (Figure 22c, 24). There was a slightly significant population of neurons in the Mup 24-Mup 25 responsive pool of neurons that also responded to N25 at a rate of 0.165% of total cells (8 responsive cells, $p=0.0404$) (Figure 22d, 24). There was no significant response however to Mup 25 alone (15 responsive cells, $p=0.691$) (Figure 24). Additionally, the N25 chimera did not activate a significant population of neurons

(16 responsive cells, p=0.3542) (Figure 24) Finally, there was a population of generalist neurons responding to all stimuli at a rate of 0.985% (60 responsive cells, p<0.0001) (Figure 22e, 24).

The lack of behavior seen from stimulation by the N25 chimera is not due to additional activation. However, there is a significant population of neurons that were activated by Mup 24 and Mup 25 that lacked a response to the chimera (Figure 22, 24). This population of neurons therefore could be required for the coding from the VNO for this stereotyped male to male aggressive response.

Discussion

The NTD of Mups is required for specialist detection in the VNO

Mouse Mups are known activate specific subsets of vomeronasal neurons depending on the identity of the Mup, however the basis behind this specialized coding was unknown. Despite the fact that Mups are highly homologous proteins, the divergences found in their sequences are sufficient to alter the activation profile in the VNO. This specialist coding could be important in differentiating activation profiles in the VNO allowing for diverse behavioral responses to different Mups. To elucidate the mechanism behind the coding in the VNO looked at the functional significance of class A and Bs, the specificity of ligand/receptor interactions, and how the VNO differentiates between intraspecies (and possibly interspecies) Mups. We manipulated this initial sensory activation in the VNO through point mutations and chimera construction of the Mup proteins to address these questions.

The highly homologous class B Mups are sufficient to activate separate subsets of VNO neurons (Figure 8, 11). In calcium imaging experiments, residues 50 and 13 are individually sufficient to activate specialist subsets of neurons (Figure 8, 11). Mup 8 contains a single polymorphic residue at position 50, while Mup 4 and Mup 13 contain a divergent residue at position 13 (Figure 9a). On examining the crystal structure of the Mups, both the residue at position 13 and the residue at position 50 are localized in the same region of the amino terminus face of the Mup (Figure 9b). In contrast to this, residue 140 in the CTD of Mups is insufficient to activate specialist neurons (Figure 12). This is an additional support for the hypothesis

that Mups activate their specialist receptors through the amino terminus face of the protein.

While the VNO has evolved a highly specialized system to detect single residue substitutions in the class B Mups, detection of the class A Mups is less refined given the higher level of divergence between the class A Mups (Figure 4). When examining the sequences of Mups, it is found that the majority of divergences are in the amino terminus (Figure 5), indicating VN receptors may be interacting with class A Mups in the same region as class Bs. In concordance with this, neurons responding to Mup 8 and Mup 25 specifically also respond in an overlapping manner with the N8 and N25 chimeras, respectively (Figure 15, 19). This is supports the hypothesis that the NTD of Mups is indeed implicated in specialist coding.

Of all of the Mups expressed in C57Bl/6J mice, Mup 24 and Mup 25 alone have their function as pheromones elucidated and characterized. These both belong to the more divergent class As, raising questions as to the evolutionary driving force behind the exquisitely tuned system that is seen in the class Bs. There are two possibilities for these two distinct classes. One possibility is that while the class A Mups serves a direct innate function such as the instigation of male to male aggression, the class B Mups has no innate functionality. Instead, the class Bs could act in conjunction with the class As to confer individuality, a function previously ascribed to Mups (Brennan, 2004; Hurst et al., 2001). The second explanation is that there is an innate behavior driven by the class B Mups that has not yet been elucidated, and that both classes of Mup have the additional function of individuality coding.

The ability of a single residue substitution to change the neuronal activation profile accorded to a class B Mup is similar to the high level of specificity seen in MHC peptide coding, another peptide shown to have pheromone activity. MHC peptides have a non-classical function as pheromones that can activate subsets of both VNO and MOE sensory neurons (Leinders-Zufall et al., 2004; Spehr et al., 2006). In females, it was found that the MHC peptides were sufficient to induce pregnancy abortion, a behavior seen when a female detects an unfamiliar male, implicating these peptides in individuality sensing (Leinders-Zufall et al., 2004). These peptide ligands have been found to activate various subsets of VNs dependent on single residue substitutions in their sequence (Leinders-Zufall et al., 2009). This involvement of such a highly tuned system in an innate process sets precedent for the Mup family to serve a similar function. Indeed, behaviors such as dominant male countermarking in response to an unknown male's urine are believed to be dependent upon Mups as opposed to volatile odor cues (Hurst et al., 2001; Nevison et al., 2003). This is an innate behavior that may require a more finely tuned detection system to account for modulations due to social status and relatedness. These proposed functions of Mups are interesting in that while Mups are sufficient for an innate behavior such as aggression, they have also been shown to act as cues to modulate behaviors that have more potential for complex signaling.

Possible mechanisms of vomeronasal detection of Mups

The mechanisms behind the initial interaction of Mups with receptors are poorly understood. To better understand how these Mups may be eliciting this initial

activation of the VNO, we can more closely look at characteristics of the VN receptors. The traditional methods used to elucidate receptor-ligand interactions via biochemical methods are not available at this time given the nature of these receptors (Bush and Hall, 2008), but we can compare the characteristics of V2Rs to systems that share known characteristics. Receptors of the V2R C family have been found to be broadly expressed in Gαo-expressing neurons, while members of the other V2R families are co-expressed in these same neurons (Martini et al., 2001). This mode of expression is similar to the gustatory system, where sensory neurons co express different receptor classes in order to detect different tastes such as sweet, sour, and bitter ligands (Konstantinidis, 2009).

Like V2R receptors, taste receptors are G-protein coupled receptors with a large extracellular amino terminus domain. These receptors interact in a heterodimeric fashion to bind with both small and large taste ligands. For example, the sweet receptor consists of a dimer between the T1R2 and T1R3 proteins. The binding of sweet proteins to the sweet receptor has been modeled after a wedge model of binding, where one protein face is implicated in binding to the extracellular domain of the receptor (Temussi, 2002).

The NTD of Mups has been implicated in activation of specialist neurons in the VNO, supporting a wedge model of activation. A possible contradiction to this model of receptor-ligand interaction however is found in Mup 3. Of the class B Mups, Mup 3 and 8 has been shown to be sufficient to activate a distinct subset of neurons (Figure 8a,i-ii). However, while Mup 8 has a polymorphic residue at postion 50 in the

NTD, in Mup 3 the two polymorphic residues reside either on the C terminus domain at position 79, or inside the hydrophobic binding region at position 56 (Figure 9a). Initially this seems to contradict the hypothesis that specialist coding is dependent upon the NTD of Mups. However, a similar effect was found in the sweet peptide MNEI, when a mutation of the hydrophobic core of the ligand led to a partial relative displacement of the outer face of the protein. This mutation significantly decreased binding of MNEI, fundamentally altering the sweet taste (Spadaccini et al., 2003). Additionally, efficient binding of the sweet protein brazzein was found to be dependent mainly on the overall shape and charge complementarities (Morini et al., 2005). Thus this mutation at position 56 in Mup 3 may also alter the overall shape of the protein, allowing for unique coding despite the lack of polymorphic residues in the amino terminus face. These factors of sweet protein binding can account for the unique activation of Mup 3 in the VNO. Both sweet and Mup protein to ligand interactions may involve a large section of the protein surface.

The basis of Mup coding in the VNO seems to be reliant on the NTD. The potential of polymorphisms in the hydrophobic region of Mups may not only change the outer structure and activation capabilities, but also how small volatiles may bind. When a Mup binds a small volatile, the Bc, Bd and c/d loop of the Mup protein shift (Timm et al., 2001). This region of the Mup includes the last beta sheet of the chimera found to induce specialist coding, as well as the first beta sheet (B_d) of the CTD (Figure 13a). This conformational change may allow Mups to activate a unique subset of neurons, extending their role beyond a transport vehicle for these small molecule

pheromones. This data supports a potential mechanism for a protein-small molecule complex to behave as a pheromone, in addition to altering Mup coding capacity with mutations in this region.

Neuronal coding of pheromones in the vomeronasal organ

Although the N25 chimera neuron activation is contained within Mup 25 specialist neuron activity, it does not account for all responsive specialist neurons (Figure 16). This lack of activity is not accounted for by the CTD of the N8 chimera, indicating that this activation loss was not due to interaction from the opposite face (Figure 20). This would indicate that while the NTD of Mup 25 was not sufficient to incorporate all Mup 25 responsive neurons, the CTD of Mup 25 fails to account for this missing population. When primary VNs are presented with Mup 24 in addition to Mup 25, Mup 8 and the N25 chimera, it is found that this 24/25 population of neurons is not accounted for by the N25 chimera (Figure 23c, 24). This 24/25 population of neurons accounts for the aberrant 25 specialist population found in the previous experiment (Figure 14b, 16). Finally, when assayed for behavior it was found that the N25 chimera was not sufficient to instigate stereotyped aggression (Figure 21).

One possible explanation for this loss in activation is that while the NTD region of the protein is required for specialist activation of neurons, there may be a larger surface area of the class A Mups involved. Given the higher divergence between class A Mups (Figure 5), the region of the receptor interacting with the Mups may need to have a broader scope. In support of this argument, the fourth strand of the beta barrel of Mup 25 is 80% divergent from Mup 8 (Figure 13a, b). The fourth strand

of the beta barrel in Mup 8 however has 100% identity between all class Bs (Figure 9). This indicates that this domain may be important in Mup25 coding, although this region was not required for Mup 8 coding.

Another possibility for the loss of behavior may be that the N25 chimera is eliciting additional neuron responses that may interrupt the Mup25 signal profile. There were no significant populations of neurons responding to both N25 and Mup 8 or N8 and Mup 25. Additionally there is no significant response of neurons to either the N8 or the N25 chimera alone; however the rate of response seems to be noticeably higher than other insignificant populations (Figure 16). To account for this, we conducted an experiment with two consecutive pulses of identical Mup stimuli (Figure 17). When perfusing an identical stimulus twice, 34.78% of responding neurons do not respond to the second consecutive pulse (Figure 17, Table 4). Although experiments were purposely conducted with different orders of perfused stimuli, post hoc analysis showed that over 60% of experiments in each stimulus set in fact had the chimera presented before the respective Mup (Table 4). When the theoretical percentage of the "first pulse" populations is then calculated for each calcium experiment, it is found that the experimental data is accurate within 0.026% (Table 4). This data reaffirms that the aberrant populations that fire in response to N8 and N25 are not real populations, and therefore would not affect behavior.

The N25 chimera was not sufficient for aggression, indicating that this loss of VNO activation is in fact required for innate behavior. The four populations of neurons that fire in response to Mup 25 stimuli are the Mup 25 specialists, Mup 24/25

generalists, generalists that respond to all Mups but 24, and generalists responding to any Mup (Figure 25b). In contrast to this, only two of these populations are activated by Mup 24, which is also sufficient for aggression: the Mup 24/25 generalists and the generalists responding to any Mup (Figure 25a). Interestingly, the N25 chimera fails to mimic either of these profiles: N25 elicits activation of Mup 25 specialists, the No 24 generalists and total generalists, but fails to account for the response to the 24/25 generalists as seen from Mup 25 (Figure 25c).

This data provides information on how the activation of the VNO can lead to specific behaviors, allowing us to better understand the implications of VNO coding on its downstream target, the AOB. While still poorly understood, the AOB has been shown to detect conspecific sex from urine, as well as detect strain differences (Hendrickson et al., 2008; Luo et al., 2003). The AOB receives input from VNs, however instead of receiving input from single glomeruli as seen in the main olfactory system, the AOB output neurons receive input from multiple VNO glomeruli (Wagner et al., 2006). Recently through new techniques to directly record the AOB, it was found that the AOB has distinct populations that respond to different sex, strain, and even predator cues (Ben-Shaul et al., 2010). How might the signaling from the VNO be contributing to this ability of the AOB to delineate between both conspecific and allospecific cues? The AOB was shown to receive input from several different VNO glomeruli, while single Mup stimuli have also shown activation of several different neuron populations. It is possible that each of the activated neuron populations in response to a stimulus is required to propagate the information from that signal (Figure

25). This is a possible explanation why loss of the 24/25 VN population in response to the N25 chimera caused a loss of stereotyped aggression.

Alternatively, the 24/25 subpopulation may be both required and sufficient for successful transmission of the aggression pheromone signal. This raises additional questions however, such as the function of the Mup 25 specialist neuron response. The behavioral data indicates that activation of the Mup 25 specialist population is not sufficient for aggression behavior in male mice. One explanation is that while both of these Mups have endogenous pheromone activity, not all wild mice express the same Mups (Robertson et al., 1997). Mup 24 was found to be expressed in most but not all male mice (Thom et al., 2008), indicating that more than one aggression Mup should be found in nature. The redundancy of the proteins function therefore may be an artifact of genetic heterozygosity in the wild mouse population, the less sensitive ligand to receptor interactions in the class As, or the evolutionary importance of male to male aggression in a social context.

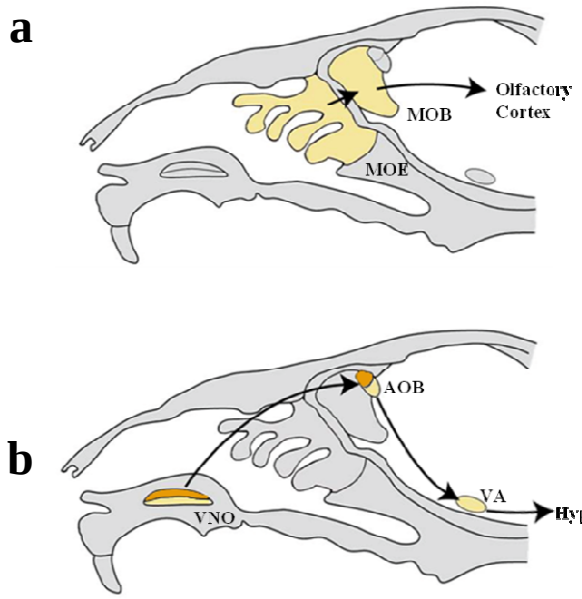
Conclusion and Future Directions

The findings of this study have elucidated the basis of neuronal coding and receptor activation in C57Bl/6J mouse Mups. The amino terminus domain of these proteins has been found to be sufficient for specialist coding in the vomeronasal organ, granting us insight into how these neurons are interacting with Mup stimuli. Because male to male aggression is the only well characterized behavior that has been shown to be elicited through Mups, this served as a good platform for investigating how the neural activation via a ligand can instigate a specific behavior. Through loss of a

specific subset of vomeronasal neurons, it was found that innate male to male aggression was also lost, effectively implicating a specific population of neurons in a specific behavior.

With these findings, we have a more complete picture of how information is encoded in the VNO. Because of the importance of the NTD in coding, constructing a new chimera including the B_d strand of Mup 25 may have the potential to rescue the behavioral response seen in Mup 25. Additionally, there are other class A Mups found in the C57Bl/6J genome that have not been found to be expressed in urine that may also possess endogenous pheromone activity. Mup 26 possesses only 11 divergent residues from Mup 25, implicating this Mup as a possible aggression-promoting pheromone. With recent advances in deep sequencing technology, we also have the opportunity to elucidate the ligand-receptor pairs seen through analyzing the mRNA transcripts present in as little as 500 picograms of material, allowing us to look at specific neurons responding to stimuli of interest. Overall, Mups have the potential to provide us with information about how sensory information is coded and transduced through neural circuits.

Figures



c

V1R	V2R
Gαi2	Gαo
Short NH2 domain	Long NH2 domain
Apical VNO	Basal VNO
Project to anterior AOB	Project to posterior AOB

Figure 1: Processing of Chemosignals through Dual Olfactory Systems

(a) Sensory neurons in the main olfactory epithelium (MOE) project to distinct glomeruli in the main olfactory bulb (MOB). Mitral cells then synapse onto the olfactory cortex. (b) Molecularly distinct sensory neurons in the vomeronasal organ (VNO) synapse onto the accessory olfactory bulb (AOB). Processes extend from the AOB to synapse onto the vomeronasal amygdala (VA) and the hypothalamus. V1R receptors (dark orange) in the apical and V2R receptors (light orange) in the basal region of the VNO project to their respective regions of the AOB as marked. (c) General characteristics of V1R and V2Rs are defined.

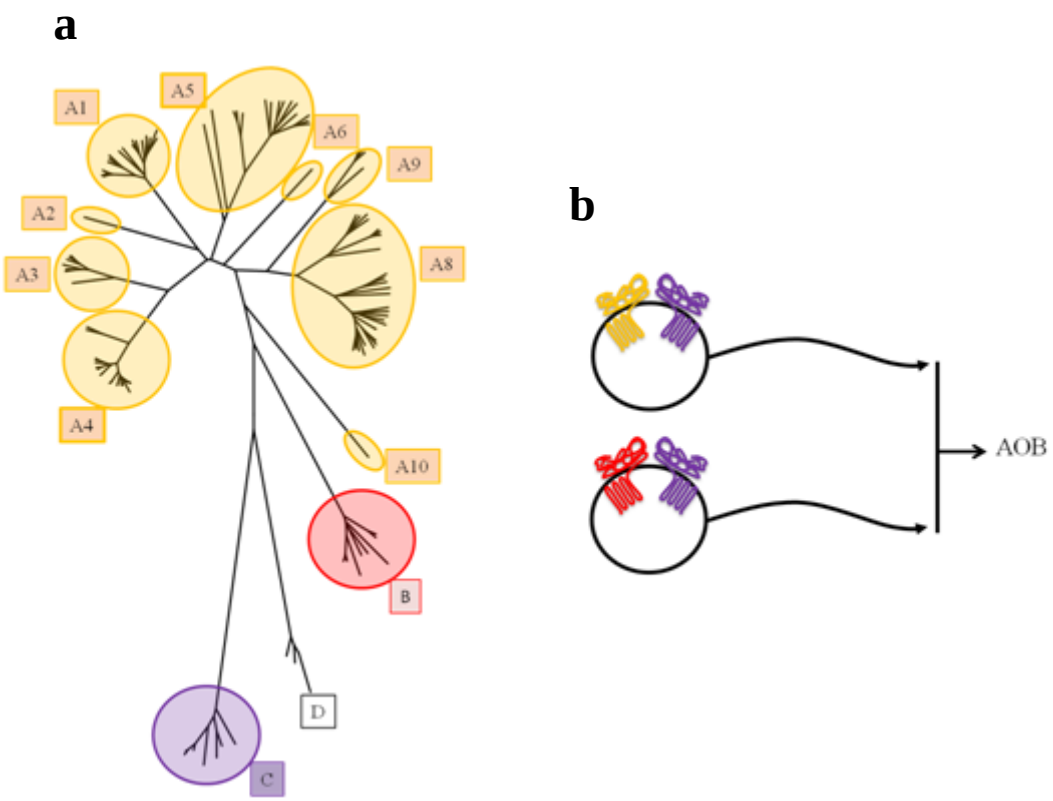


Figure 2: Heterologous Expression of V2Rs in the VNO.

(a) A phylogenetic tree of the V2R class. The V2Rs consist of over 120 open reading frames that can be organized into 12 clades. The three families known to be expressed in the neurons of the basal VNO are highlighted – members of the "A", "B" and "C" families. (b) Receptors of the V2R C family (violet) are ubiquitously expressed in Gαo-expressing neurons. Members of both the A (orange) and B (red) family of receptors are co-expressed with the C family in basal VNs.

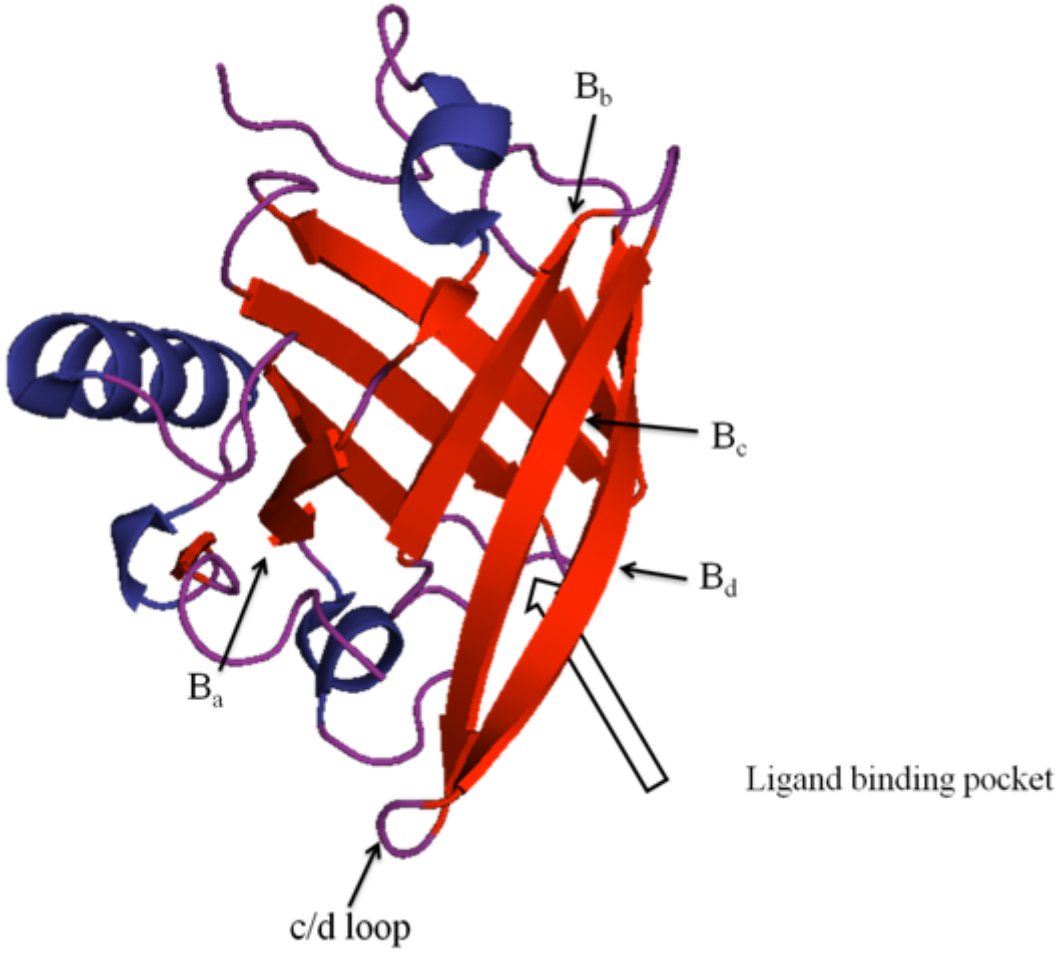


Figure 3: Crystal Structure of the Major Urinary Protein.

The structure of Mups consists of an eight stranded beta barrel, forming a hydrophobic binding pocket known to bind small volatile ligands. Regions of the protein colored purple indicates protein loops, red indicates beta sheets while blue indicates alpha helices. The first five strands of the beta barrel (B_{a-d}) are marked, as well as the c/d loop. The ligand binding pocket is indicated with a white arrow.

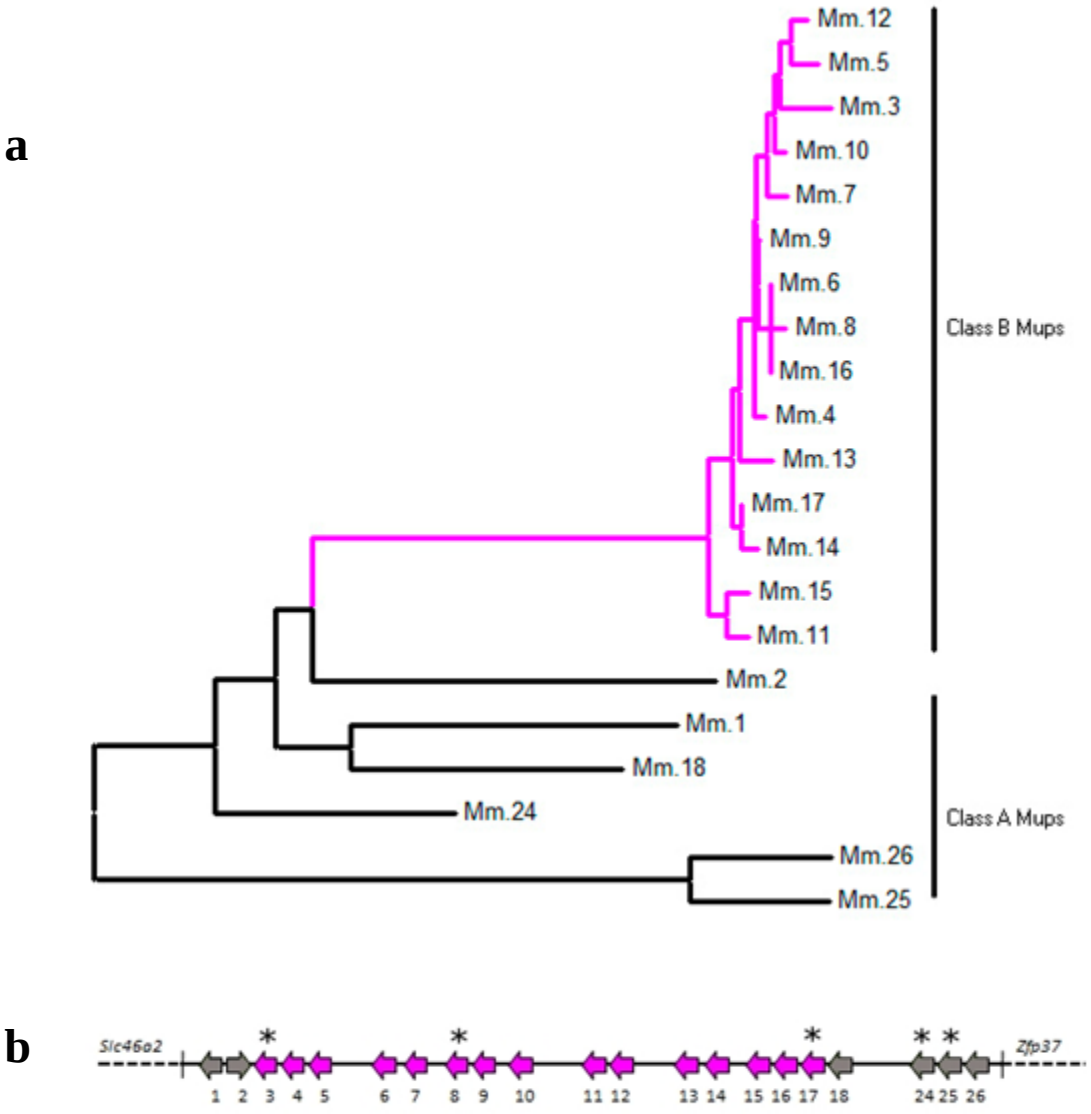


Figure 4: The C56Bl/6J Mouse Mups.

(a) Phylogenetic analysis of C57Bl/6J Mups. There is a more divergent class A family and a less divergent class B family. (b) Position of Mups on chromosome 4. Class B Mups are purple, class A Mups are grey. C57Bl/6J Mups known to be expressed in male mouse urine are indicated with an asterisk.

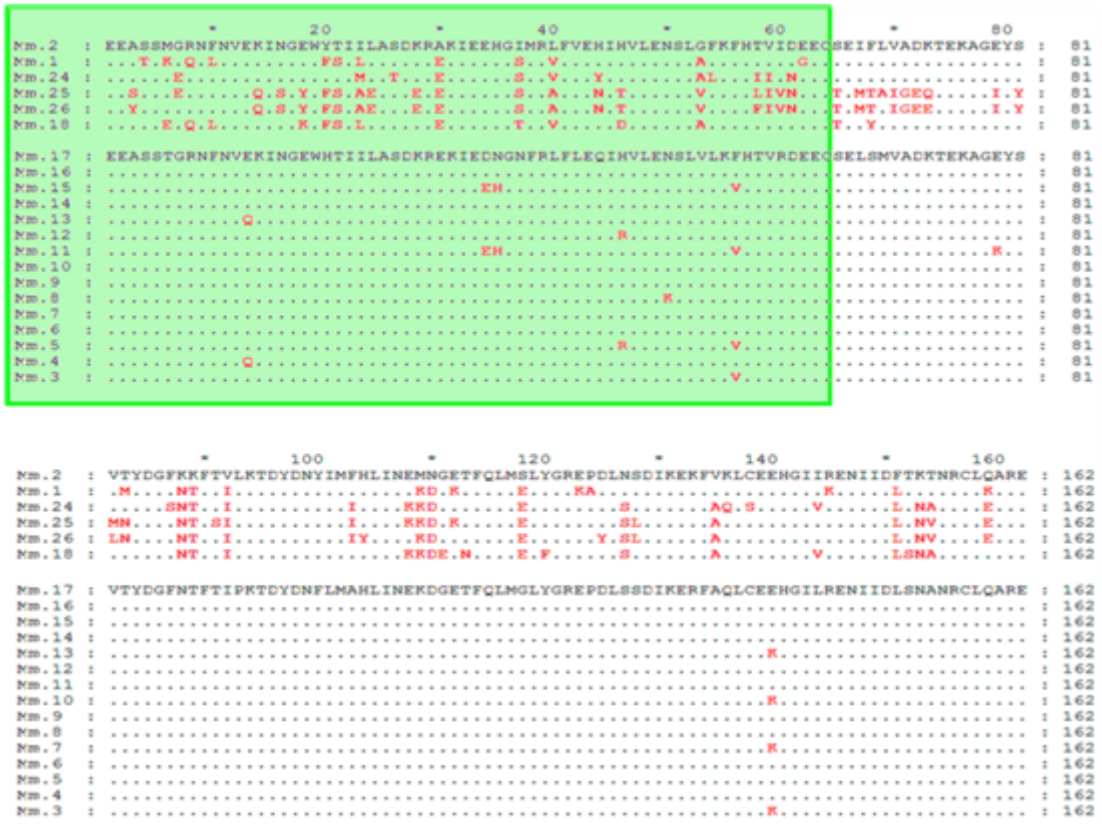


Figure 5: Alignment of Class A and B Mups.

Primary protein sequence of all of the Mups in the C57Bl/6J genome is shown divided into two classes. The class A sequences are aligned in the top and the class B sequences are in the bottom. Polymorphic residues specific to each class are highlighted in red. The amino terminus domain (NTD) used in the construction of Mup chimeras is highlighted in green.

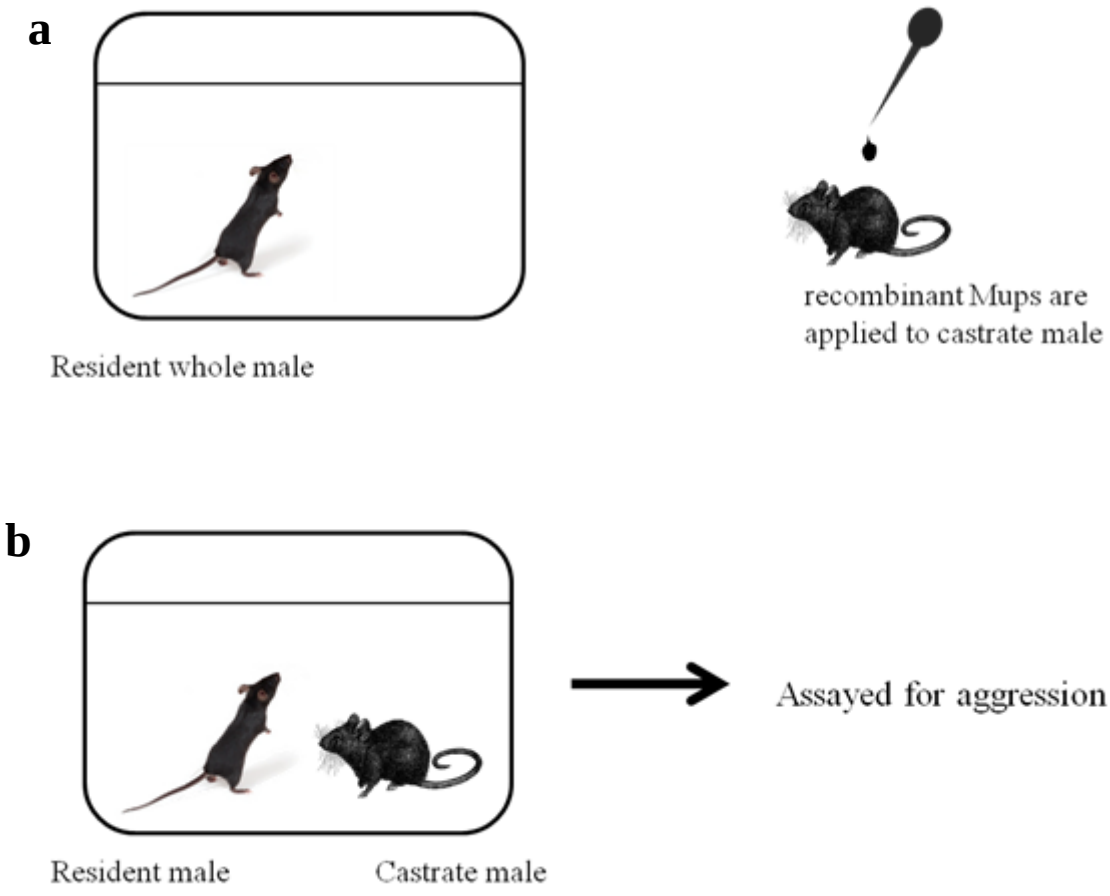


Figure 6: Experimental Setup of Aggression Assay.

Male C57Bl/6J mice were isolated for at least three weeks (a). Castrated male mouse was coated with 40 ul of stimulus (a) and placed in the cage of the intact male mouse for the duration of the assay (b) (10 minutes). Mice were videorecorded and assayed for the total duration of aggressive behavior.

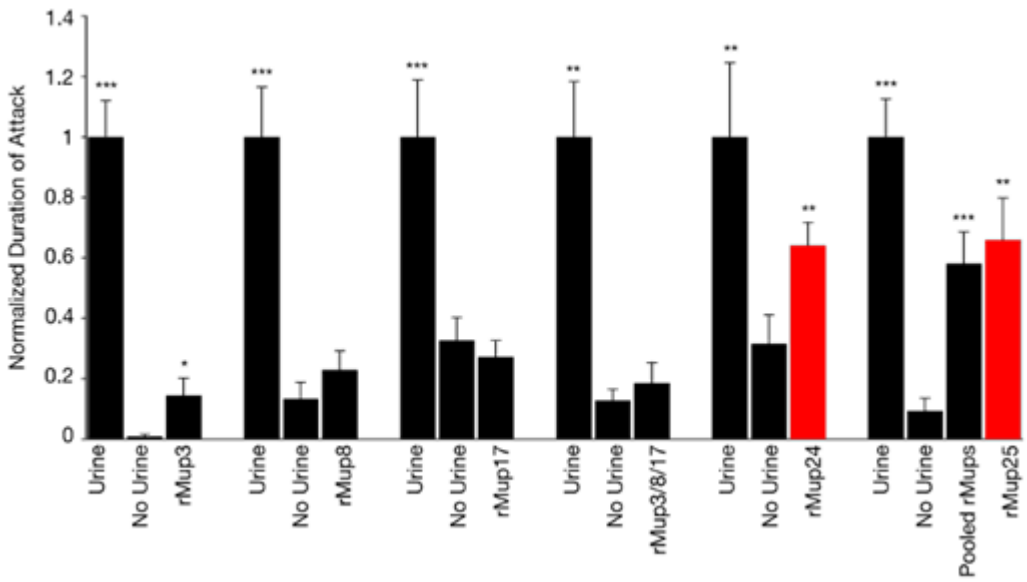


Figure 7: Mup 24 and 25 are Individually Sufficient for Stereotyped Aggression.

Duration of aggression (normalized to response in urine) in adult C57BL/6J resident males by a castrated mouse swabbed with individual or grouped Mups (mean ± s.e.m.). n(Urine) = 6-51, n(No Urine) = 6-26, n(Test) =15-44. The individual Mups that were shown to be capable of endogenous pheromone activity are highlighted in red. P values calculated by one tailed Student's t-Test compared to No Urine controls in each experimental set. *** $p<0.001$, **$p<0.01$, *$p<0.05$

Figure 7, in full, is adapted from work done by Angeldeep Kaur and Tobias Marton. This work is currently being prepared for submission.

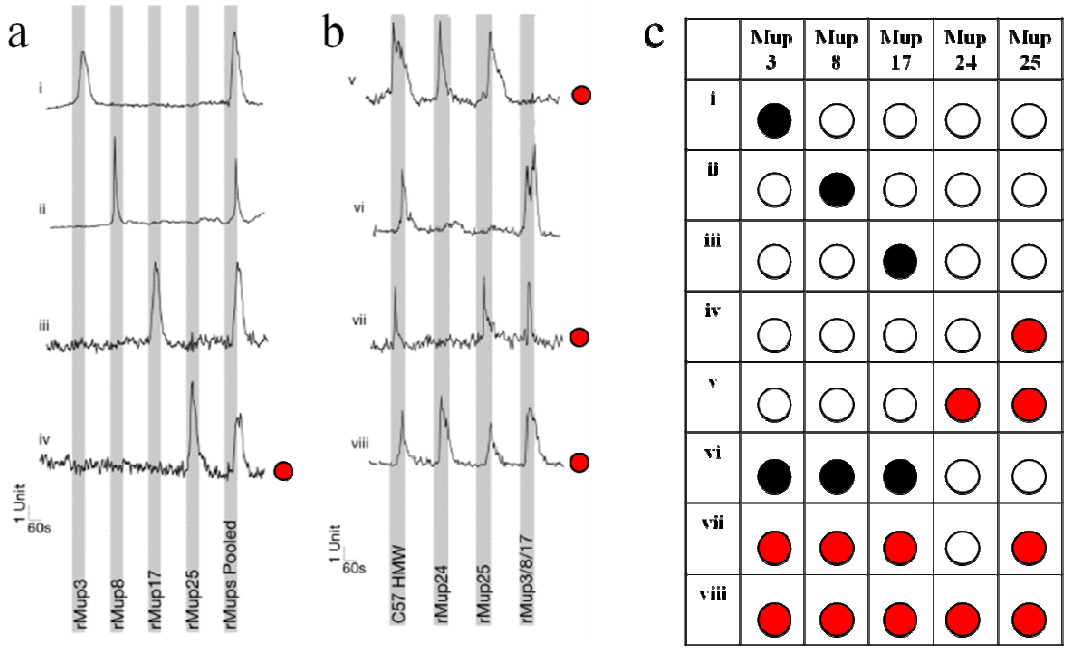


Figure 8: Mup 24 and 25 Activate Specific Subsets of VNO Neurons.

Single representative traces of eight neuron types observed upon activation by Mup 3, 8, 17, 24 and 25. Traces activated by aggression-promoting Mups are indicated with a red dot. (a) Specialist neuron traces. i. Cell responding to Mup 3. ii. Cell responding to Mup 8. iii. Cell responding to Mup 17. iv. Cell responding to Mup 25. (b) Generalist neuron traces. v. Cell responding only to class A Mups 24 and 25, vi. Cells responding specifically to class B Mups 3, 8 and 17. vii. Cell responding to all Mups except Mup 24. viii. Cell responding to all Mups. (c) Eight subset of neurons are activated by various Mup stimuli, labeled i-viii. The populations of neurons sufficient activated by aggression-promoting Mups is highlighted in red. Adapted from Marton et al, data not published.

Figure 8, in full, is adapted from work done by Angeldeep Kaur and Tobias Marton. This work is currently being prepared for submission.

a

	E13	D35	N36	H46	N50	F56	E79	E140
3						V		K
4	Q							
5				R		V		
7								K
8					K			
11		E	H			V	K	
12				R				
13	Q							K
15		E	H			V		
17								

b

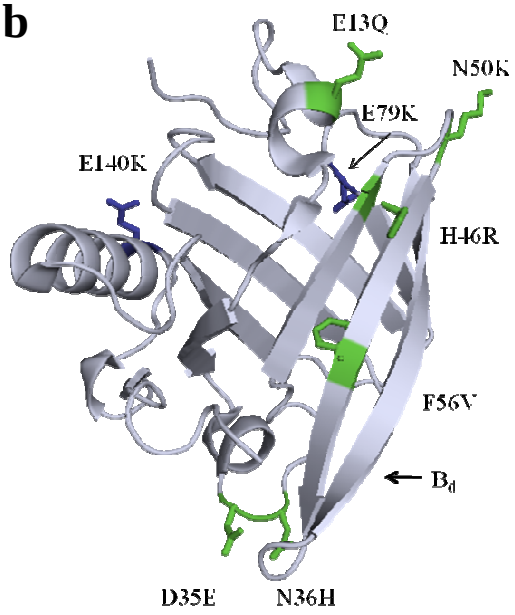
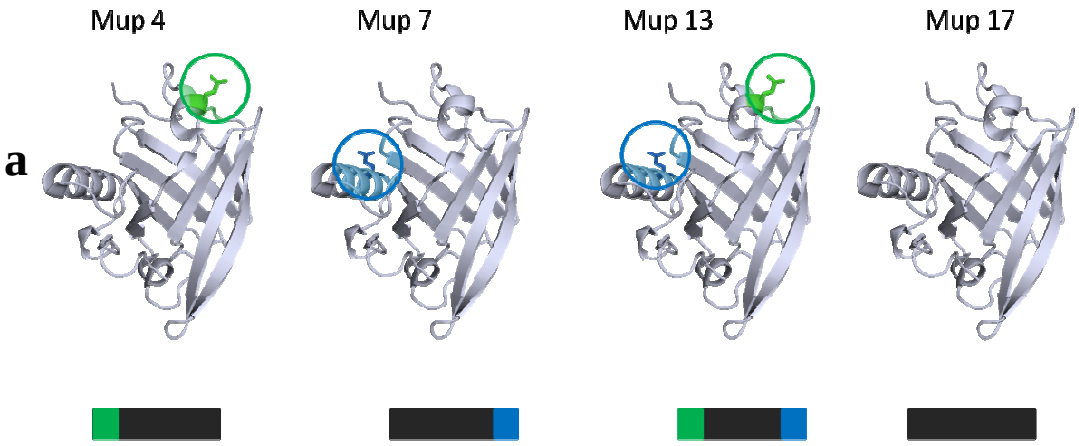


Figure 9: Positions of Class B Mup Polymorphisms.

Each of the eight class B polymorphisms in the C57Bl/6J mouse genome are shown on the crystal structure of Mups. (a) The positions of the class B polymorphisms are displayed on a chart, with the Mups shown to activate specialist neurons highlighted in grey. In (b), NTD polymorphisms are shown in green, CTD polymorphisms are shown in blue. The B_d sheet of the beta barrel is indicated as for reference.



b

		E13Q		Q140K	
Mup 4					
Mup 7					
Mup 13					
Mup 17					

Figure 10: Experimental Design of the Class B Calcium Imaging Experiment.

Each of the class B Mups perfused during the following calcium imaging experiment (Figure 11, 12) are shown above (a). Residue 13 and residue 140 are divergent from the Mup 17 consensus sequence. NTD mutations are highlighted in green, and CTD mutations are highlighted in blue. (b) Representation of polymorphic residues of Mups 4, 7, 13, and 17 shown in a table. Mups possessing the indicated substitutions are colored with either green or blue in the appropriate region.

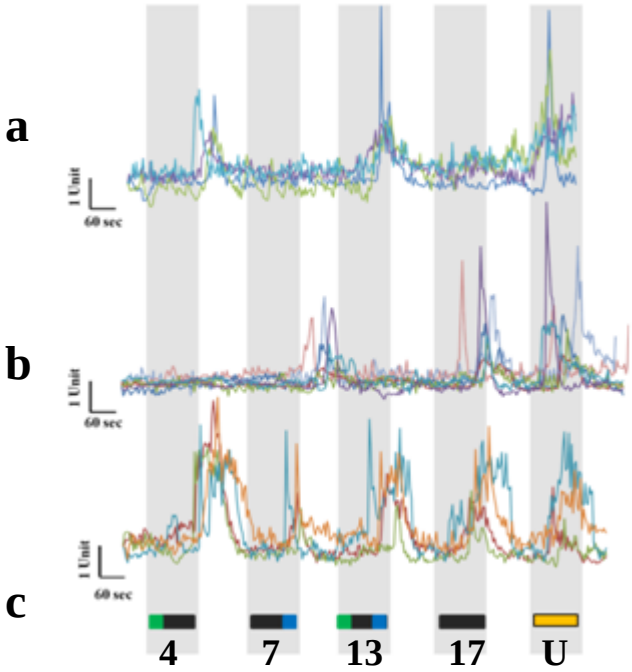


Figure 11: Representative Traces of Neurons Responding to Class B Mups.

(a) Specialist neurons respond to the K13Q mutation in Class B Mups 4 and 13. (b) Mup 17 specialist neurons fail to distinguish the E140K mutation between Mups 7 and 17. (c) Generalist neurons respond to all Mup stimuli indiscriminately. Scale bars denote the minimum spike above baseline to be considered noise (y-axis) and the timescale (x-axis).

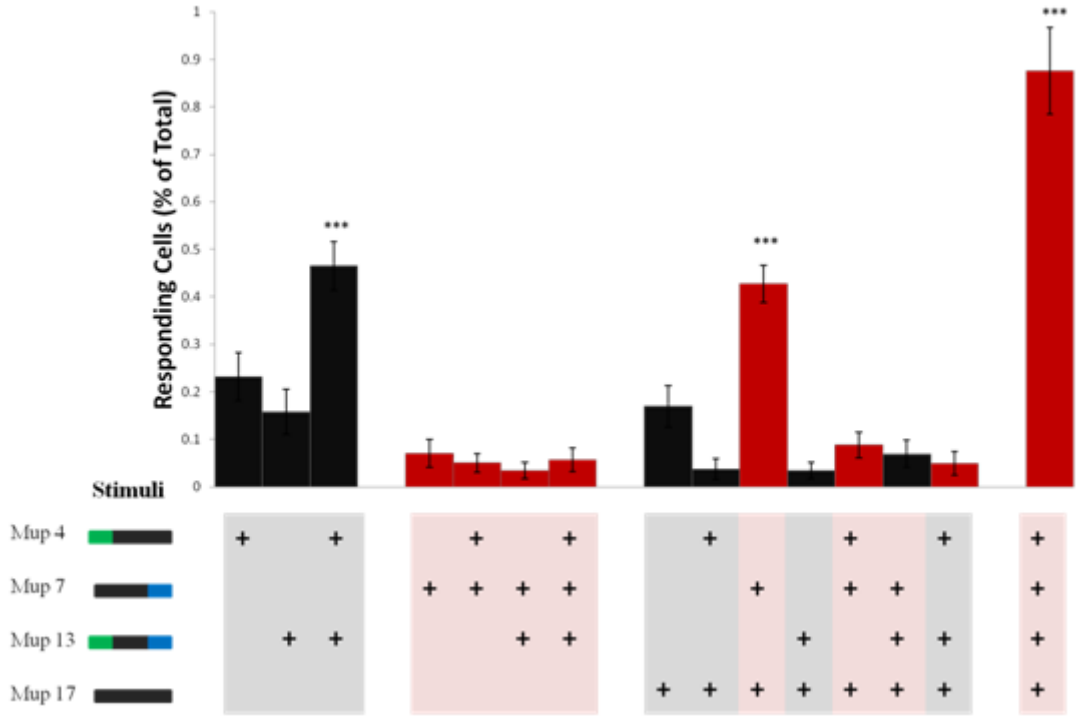


Figure 12: The K13Q Mutation is Sufficient for a Specialist Neuron Response.

Mup 4 and Mup 13 contain a K13Q mutation (green bar), while Mup 13 and Mup 7 contain a E140Q mutation (blue bar). Neurons were scored out of total cells (10339 imaged, n=39). Only 3 significant neuron populations were observed when compared to maltose binding protein (MBP) controls (Mann-Whitney-U test, n=34). These populations consisted of Mup 17 specialist neurons unable to distinguish Mup 7 (44 responsive cells, $p<0.0001$), neurons that responded indiscriminately to both Mup 4 and Mup 13 (43 responsive cells, $p<0.0001$), and generalist neurons that responded to all four stimuli (83 responsive cells, $p<0.0001$). Responders that contained Mup 7 in their activation profiles are highlighted in red, displaying a lack of any Mup 7 specialist response. *** $p<0.001$.

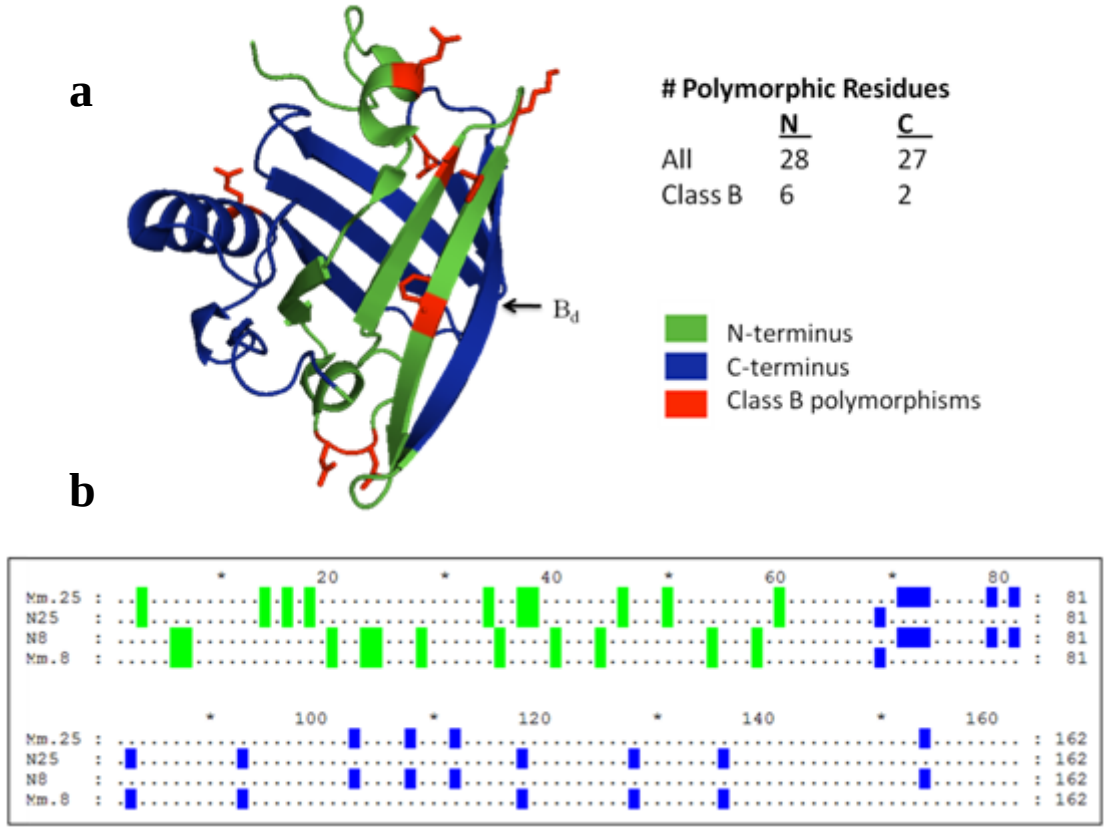


Figure 13: Alignment of Mup 8, Mup 25, N8 and N25.

(a) The crystal structure of Mups is displayed above, with the NTD region of the chimera highlighted in green, the CTD in blue, and class B polymorphisms highlighted in red for reference. The number of polymorphic residues between the class A and class B Mups is indicated to the right. The B_d beta sheet is indicated for reference. (b) All polymorphic residues in the amino terminus domain of the Mups are highlighted in green, and all carboxyl terminus polymorphisms are highlighted in blue. There are 25 divergent residues in the NTD, consisting of 39% of the protein, and 27 divergent residues in the CTD, consisting of 61% of the protein sequence.

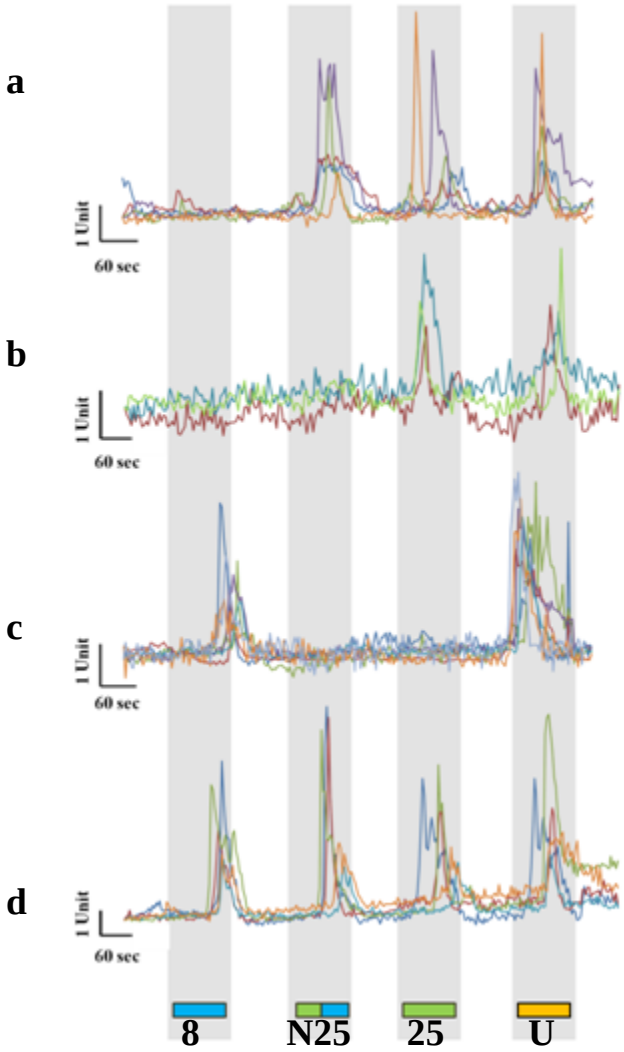


Figure 14: Representative Traces of Neurons Responding to the N25 Chimera.

(a) Neurons fail to discriminate between Mup 25 and N25 pulses. (b) There is a small, slightly significant population of neurons that respond to Mup 25. (c) Neurons respond to Mup 8 in a specialist manner. (d) Mup generalist neurons are found to respond to the N25 chimera as well. Scale bars denote the minimum spike above baseline to be considered noise (y-axis) and the timescale (x-axis).

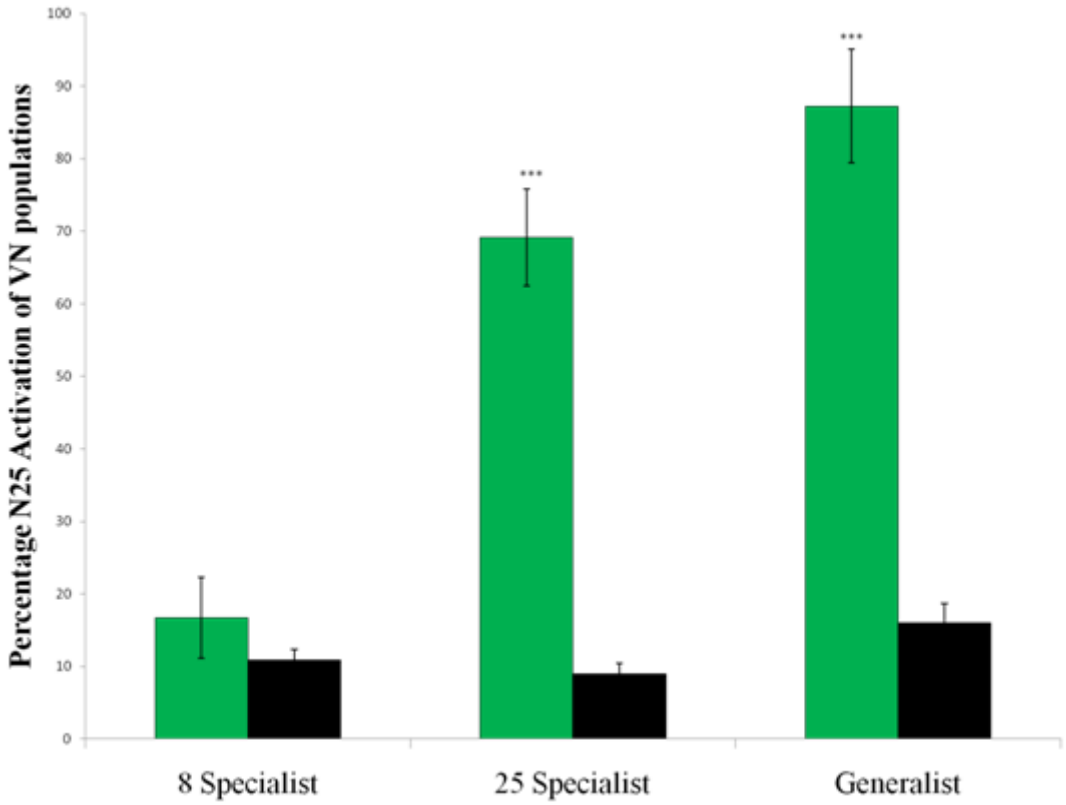


Figure 15: The N25 Chimera Activates Mup 25 Specialist Neurons.

The percent response of the N25 Chimera to the Mup 8 specialists, Mup 25 specialists, and Mup generalist pools of neurons is shown above. Neurons were counted if they responded to each of the pools above regardless of the N25 response. The percent of neurons that also responded to N25 was then calculated. These values were normalized to MBP negative controls. The difference between the two activation pools was assayed for significance using a Mann-Whitney U statistical test. The N25 chimera accounted for 16.7% of Mup 8 specialist neurons ($p=0.2846$, ns), 69.1% of Mup 25 specialist neurons ($p<0.0001$), and 87.3% of Mup generalists ($p=0.0001$). $n=17$. *** $p<0.0001$.

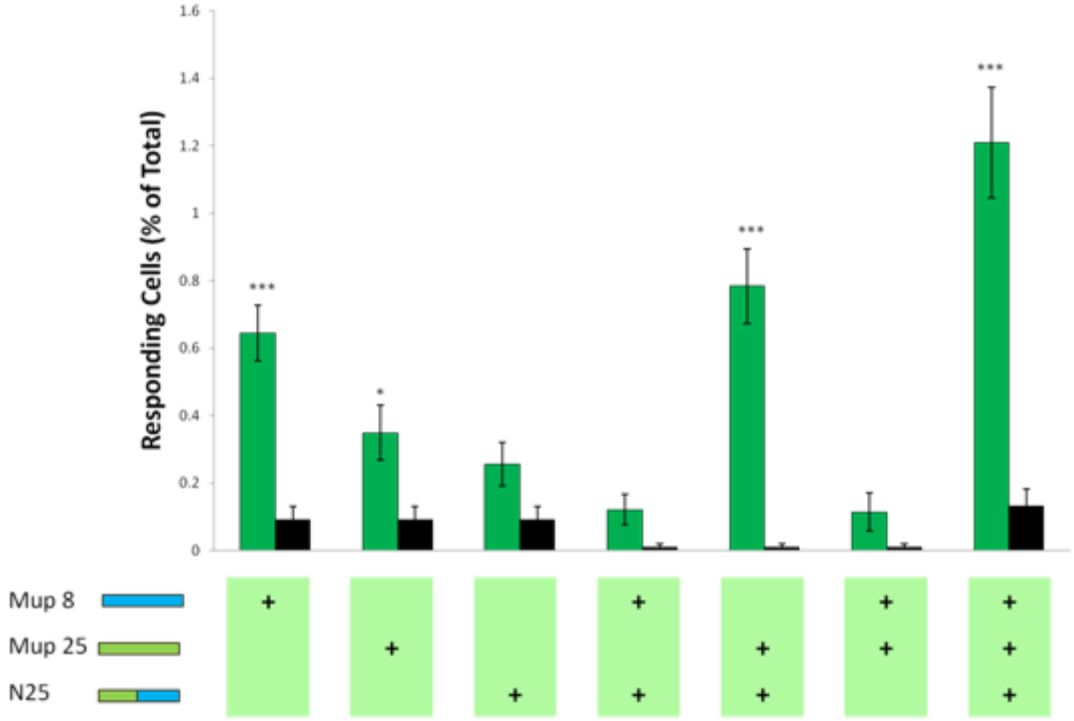


Figure 16: The NTD of Mup 25 is Sufficient for Specific Neuron Activation.

Dissociated primary neurons were perfused with stimuli consecutively. The populations of neurons responding to differential sets of stimuli of total urine responsive cells are shown above (4959 imaged, n=21). 4 significant neuron populations were observed when compared to maltose binding protein controls, displayed as black bars above (Mann-Whitney-U test, n=34). These populations consisted of Mup 8 specialist neurons (32 responsive cells, $p<0.0001$), Mup 25 responsive neurons (17 responsive cells, $p=0.0198$), Mup 25 responsive neurons that also respond to the N25 chimera (38 responsive cells, $p<0.0001$), and Mup generalist neurons that responded to all three stimuli (62 responsive cells, $p<0.0001$). ***$p<0.001$, *$p<0.05$.

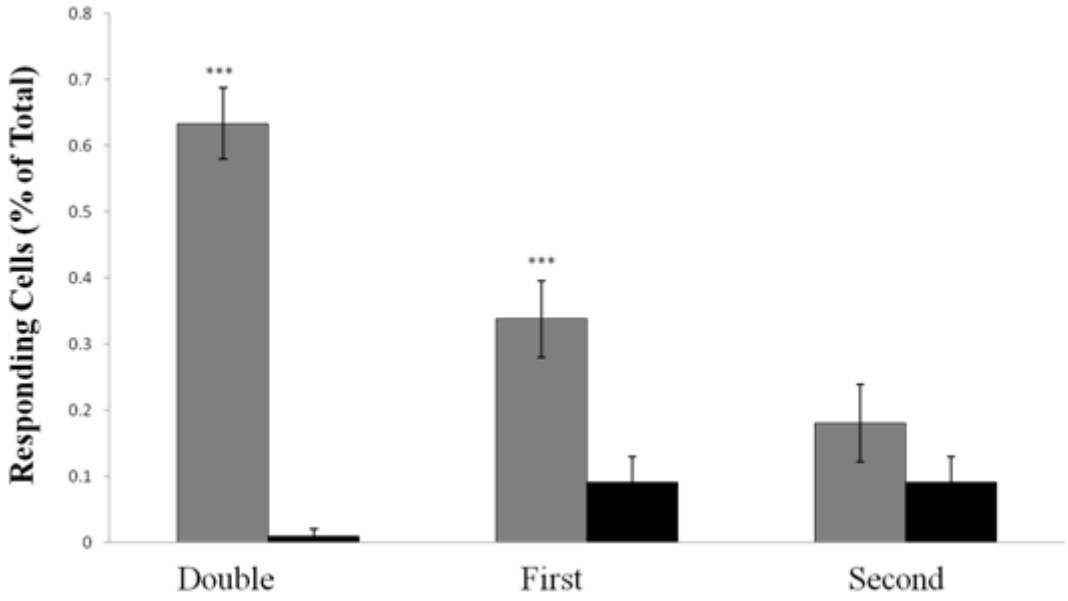


Figure 17: Double Pulse of Recombinant Mups shows a Significant Dropoff Rate.

Neurons responding to an identical stimulus pulsed twice were assayed for the amount of dropoff (a) (grey bars, mean ± s.e.m, n=21). P-values were determined by a pair wise Mann-Whitney test against Maltose Binding Protein controls (black bars, n=34). After responding to the first pulse of the stimuli, 34.78% of neurons will not respond to the second stimuli (First responder bar, p=0.0004). Neurons that respond to the second pulse of stimuli but not to the first are not significantly above noise (p=0.1645). *** p< 0.001.

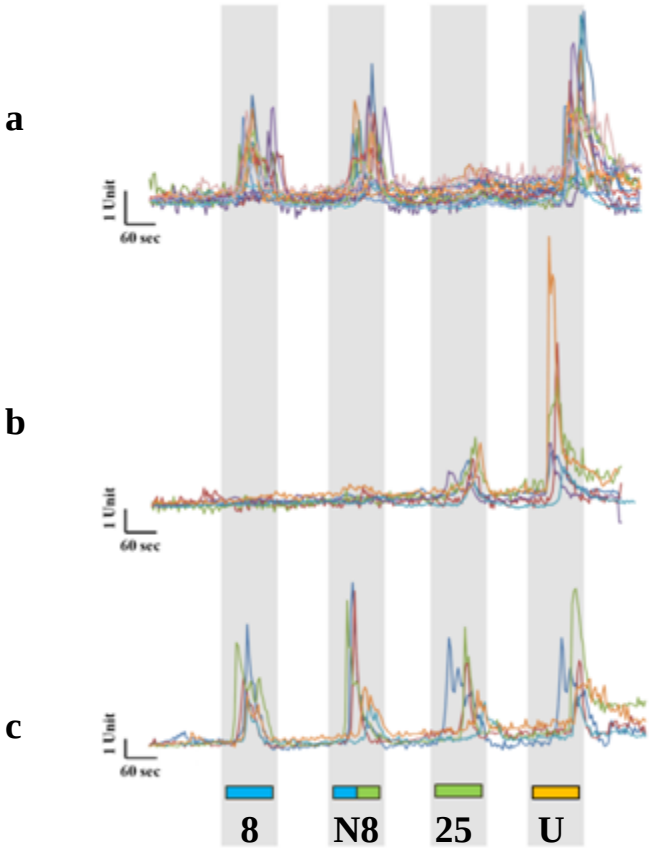


Figure 18: Representative Traces of Neurons Responding to the N8 Chimera.

(a) The NTD of the N8 chimera activates Mup 8 specialist neurons. (b) Neurons responding to Mup 25 in a specialist manner fail to activate in the presence of N8. (c) Mup generalist neurons responding to all four stimuli. Scale bars denote the minimum spike above baseline to be considered noise (y-axis) and the timescale (x-axis).

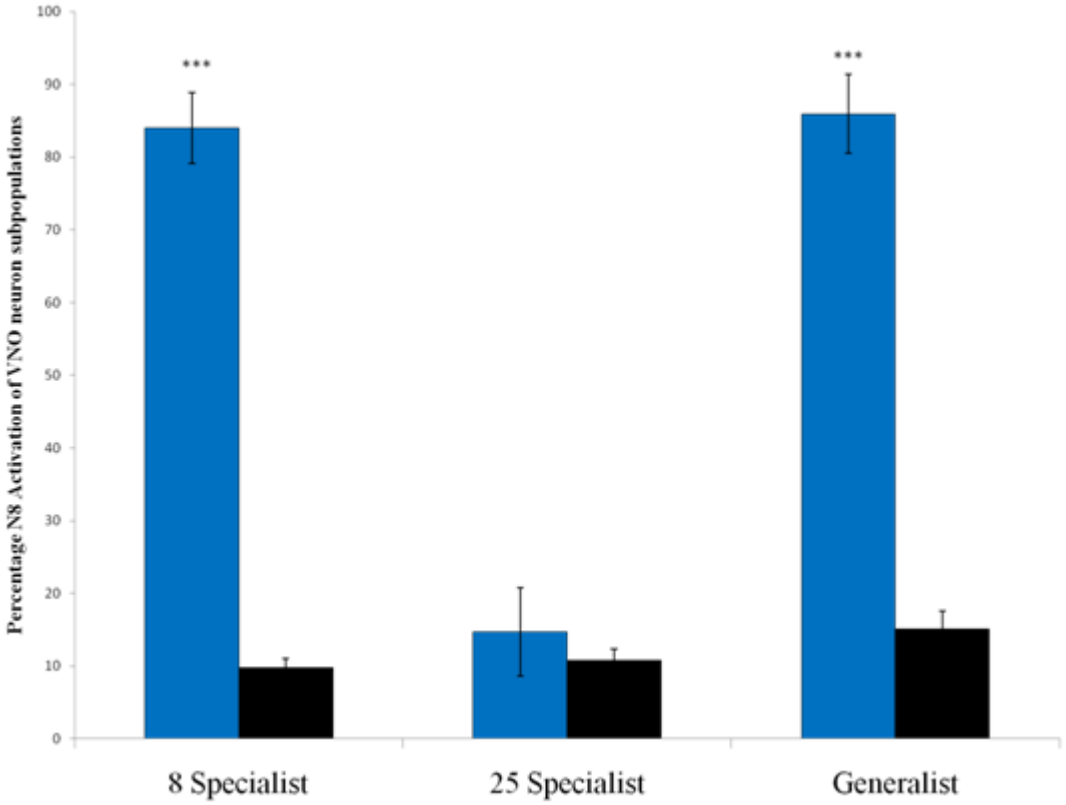


Figure 19: The N8 Chimera Activates Mup 8 Specialist Neurons.

The percent response of the N8 Chimera to the Mup 8 specialists, Mup 25 specialists, and Mup generalist pools of neurons are shown above. The percent of neurons that also responded to N8 was calculated, and then compared to MBP negative controls. The difference from the negative control was then assayed for significance using a Mann-Whitney U statistical test. The N8 chimera accounted for 84.0% of Mup 8 specialist neurons ($p<0.0001$), 11.4% of Mup 25 specialist neurons ($p=0.7155$, ns), and 85.9% of Mup generalists ($p=0.0001$). $n = 20$, *** $p<0.0001$.

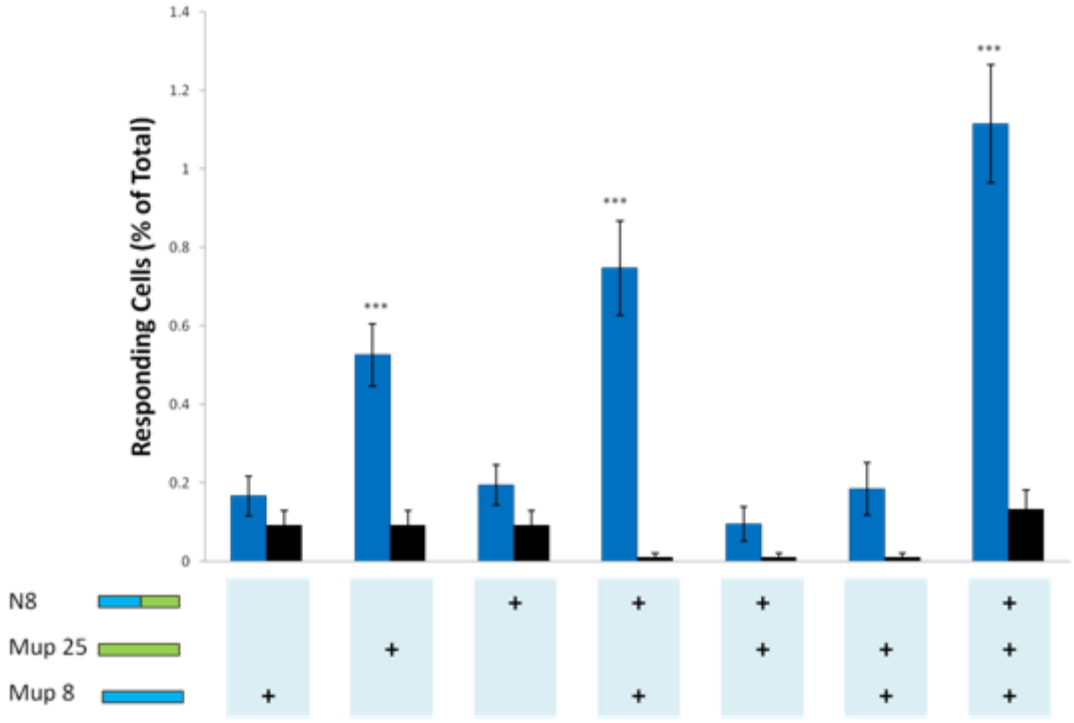


Figure 20: The NTD of Mup 8 is Sufficient for Specific Neuron Activation.

Dissociated primary neurons were perfused with stimuli consecutively. The populations of neurons responding to differential sets of stimuli of total urine responsive cells are shown above (5965 imaged, n=27). 3 significant neuron populations were observed when compared to maltose binding protein controls, displayed as black bars above (Mann-Whitney-U test, n=34). These populations consisted of Mup 25 specialists (34 responsive cells, $p=0.0001$), Mup 8 specialists responding to the N8 chimera (42 responsive cells, $p<0.0001$), and Mup generalist neurons that responded to all three stimuli (61 responsive cells, $p<0.0001$). ***$p<0.001$

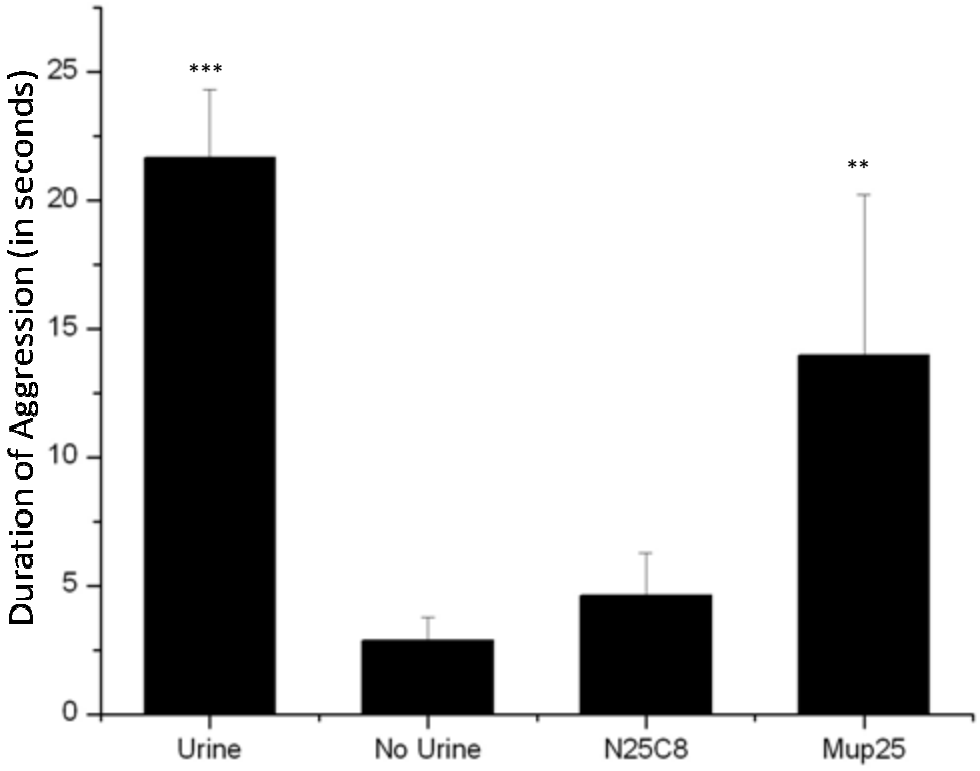


Figure 21: The N25 Chimera is not Sufficient for Stereotyped Aggression

C57Bl/6J mice were used in a 10 minute aggression assay to determine the aggression-promoting ability of the N25 chimera. Significance between aggression durations was calculated with a Mann-Whitney-U statistical test. Urine was used as a positive control, and mice elicited aggressive behaviors for an average of 21.66 seconds (n=25, ±2.655 s.e.m., $p<0.0001$). This was significantly above the negative control of male mice paired with castrates (2.863 seconds, n=17, ±0.921 s.e.m). The N25 chimera did not elicit aggression significantly above noise (4.630 seconds, n=21, ±1.637 s.e.m). Mup 25 was sufficient to promote aggression behavior significantly greater than the blank castrate (13.976 seconds, n=6, ±6.222, $p=0.00294$). ***$p<0.001$, **$p<0.01$, *$p<0.05$

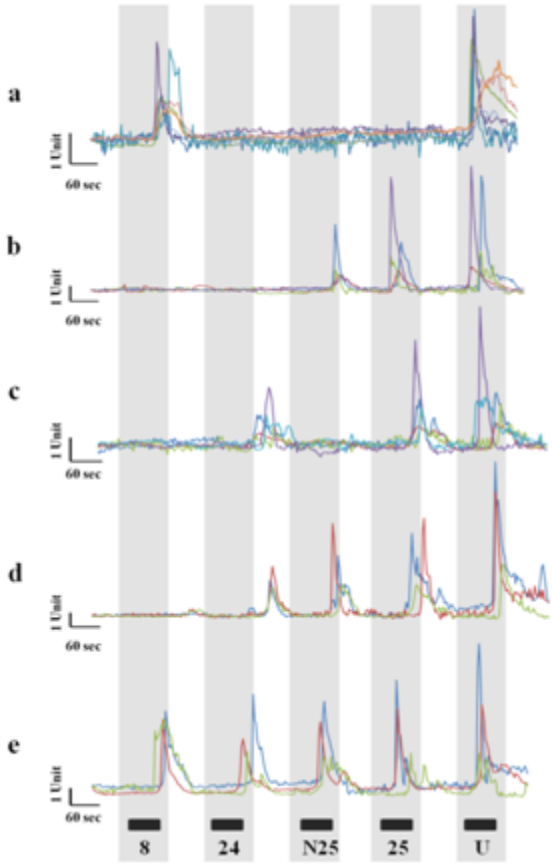


Figure 22: Representative Traces of the N25 Chimera and Mup 24 Activation.

Representative traces of (a) 8 specialist and (b) 25 specialist neurons. The Mup 8 specialists will not fire in response to the N25 chimera, but the Mup 25 specialists do. (c) The majority of 24/25 specialist neurons are not activated by N25, but (d) there is a small subset of neurons that also respond to N25. (e) Generalist neurons that respond to all stimuli. Scale bars denote the minimum spike above baseline to be considered noise (y-axis) and the timescale (x-axis).

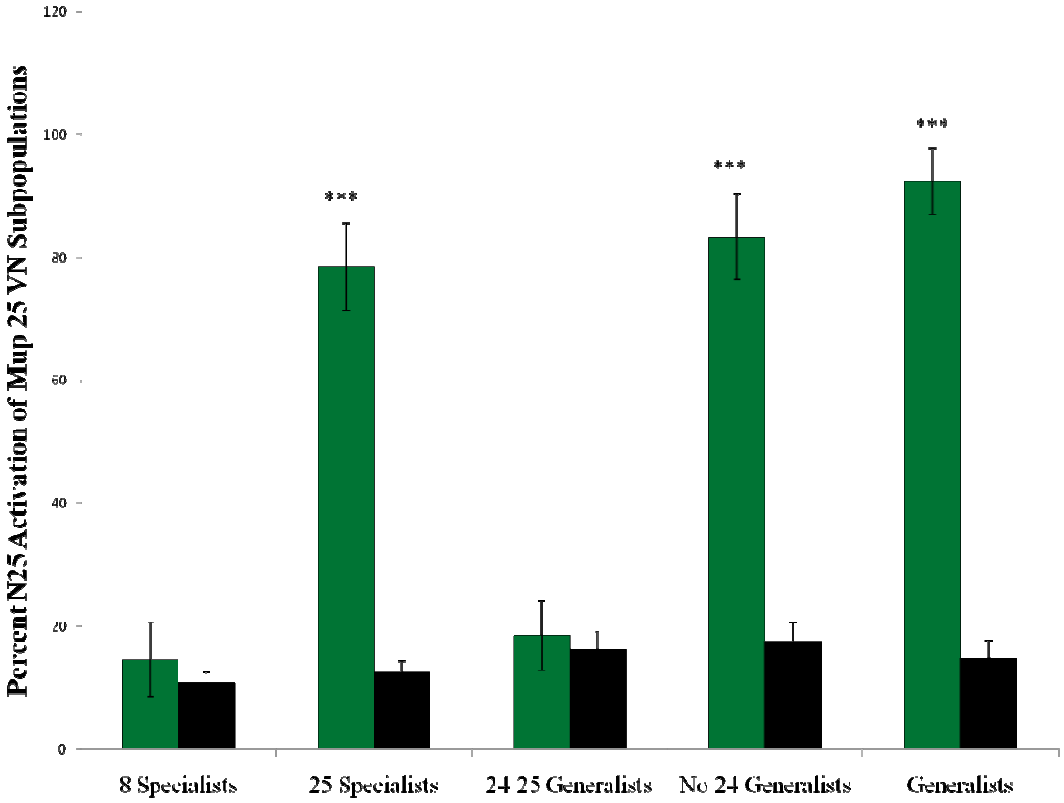


Figure 23 – The N25 Chimera does not Activate Mup 24/25 Generalist Neurons

The percent response of the N25 Chimera to the four Mup 25 responsive pools of neurons as well as the Mup 8 specialist pool is shown above. Neurons were counted if they responded to each of the pools above regardless of the N25 response. The percent of neurons that also responded to N25 was then calculated. These values were normalized to MBP negative controls. The difference between the two activation pools was assayed for significance using a Mann-Whitney U statistical test. The N25 chimera accounted for 14.6% of Mup 8 specialists ($p=0.1031$, ns), 78.4% of Mup 25 specialists ($p<0.0001$), 18.4% of Mup 24/25 generalists ($p=0.5029$, ns), 83.3% of No 24 generalists ($p<0.001$), and 92.4% of Mup generalists ($p<0.0001$). n = 19, ***$p<0.0001$.

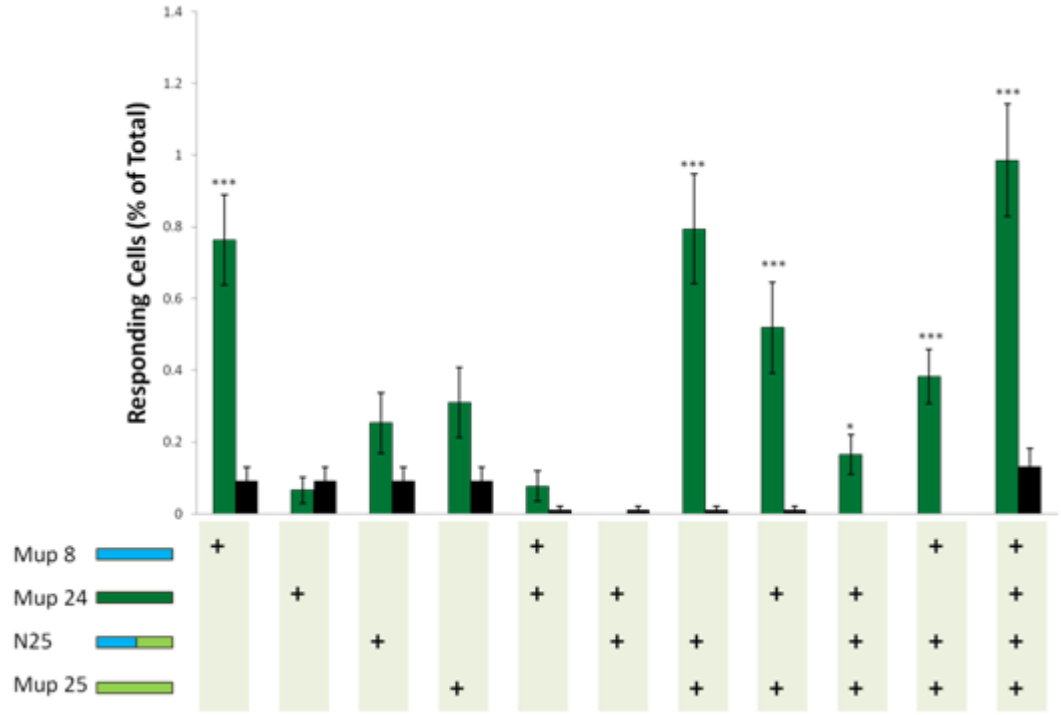


Figure 24: The N25 Chimera does not Account for 24/25 Neurons.

Dissociated primary neurons were perfused with stimuli consecutively. The populations of neurons responding to differential sets of stimuli of total responsive cells are shown above (6219 imaged, n=25). 6 significant neuron populations were observed when compared to maltose binding protein controls, displayed as black bars above (Mann-Whitney-U test, n=34). These populations consisted of Mup 8 specialists (42 responsive cells, $p<0.0001$), Mup 25 specialists responding to the N25 chimera (38 responsive cells, $p<0.0001$), 24/25 generalists (24 responsive cells, $p<0.0001$), 24/25 generalist neurons also responding to the N25 chimera (8 responsive cells, $p=0.0300$), No Mup 24 generalists also responding to the N25 chimera (30 responsive cells, $p<0.0001$), and Mup generalists also responding to the N25 chimera (60 of 573 total responsive cells, $p<0.0001$). ***$p<0.001$, *$p<0.05$

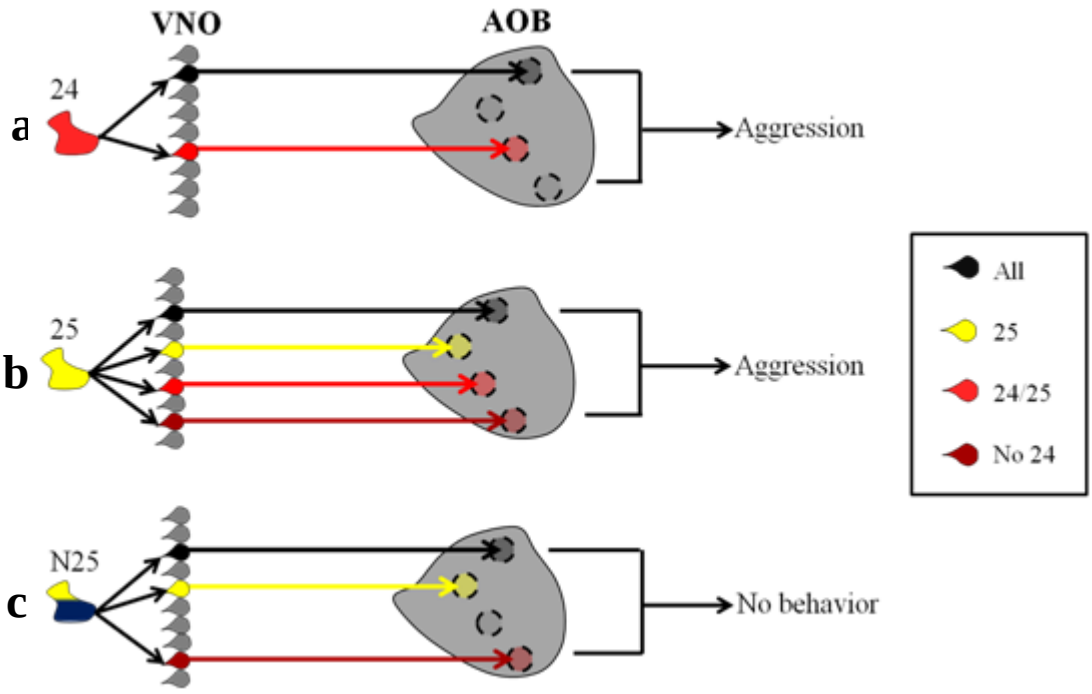


Figure 25: N25-Activated Neuron Populations are not Sufficient for Aggression

A schematic model for VNO activation is shown above. (a) Response profile of neurons to Mup 24. Mup generalists and Mup 24 specialists are activated. (b) Response profile of neurons to Mup 25. Mup generalists, 25 specialists, 24/25 generalists, and No 24 generalists are activated. (c) Response profile of VNO neurons to the N25 chimera. Mup generalists, Mup 25 specialists and No 24 generalists are activated. The 24/25 pool of neurons is not activated by the N25 chimera, and this profile of activation is not sufficient for a behavioral response.

Tables

Table 1: Accession Numbers for C57Bl/6J Mups

Asterisks mark the Mups known to be expressed in C57Bl/6J urine. The other Mups listed are the ones referred to in this study. Mups not used in this study are not shown.

Protein name	**DDBJ/EMBL/GenBank accession**
Mup 3*	EU882230
Mup 4	BK006642
Mup 7	BK006648
Mup 8*	EU882231
Mup 13	BK006661
Mup 17*	EU882232
Mup 24*	EU882234
Mup 25*	EU882235

Table 2: Major Urinary Protein PCR Primers.

Primers used for Mup cloning from cDNA prepared from male C57Bl/6J mouse VNO RNA.

Primer Name	**Primer Sequence**
Mup 3 Forward	GGATCCATGAAGATGCTGCTGCTG
Mup 8 Forward	GGATCCATGAAGATGATGCTGCTG
Mup 17 Forward	GGATCCATGCTGTTGCTGCTGTGT
Class B Reverse	AAGCTTTCATTCTCGGGCCTGGAG
Mup 24 Forward	GGATCCATGAAGCTGCTGGTGCTG
Mup 24 Reverse	AAGCTTTCATTCTCGGGCCTCAAG
Mup 25 Forward	GGATCCATGAAGCTGCTGCTGCCG
Mup 25 Reverse	AAGCTTTCATTCTCGGGCCTCGAG

Table 3: Mutagenesis Primers used in Class B Mup Design.

BMup_E13Q_F	5’-GGGAAGGAACTTTAATGTACAAAAGATTAATGGGGAATGGC-3’
BMup_E13Q_R	5’-GCCATTCCCCATTAATCTTTTGTACATTAAAGTTCCTTCCC-3’
BMup_E140K_F	5’-GGTTTGCACAACTATGTGAGAAGCATGGAATCCTTAGAG3’
BMup_E140K_R	5’-CTCTAAGGATTCCATGCTTCTCACATAGTTGTGCAAACC-3’

Table 4: Calculations of Theoretical Dropoff

Using the known drop off rate of neurons responding to identical consecutive stimuli (65.22%), and the percentage of experiments where the chimera was pulsed first (%N Pulsed First column), the theoretical percent of total neurons responding can be calculated (Theoretical First Pulse). 2X Responders are the percent of neurons that responded to both N8 and Mup 8 or N25 and Mup 25, while Experimental First Pulses are neurons that responded to the chimera only. Total Responses are the combined percentage responders of both 2X Responders and the Experimental First Pulse. This number was multiplied by the theoretical percent of neurons that will respond only once (34.78%) and the percent of experiments where the chimera was pulsed first (last column). This value is called the theoretical first pulse. The theoretical populations are comparable to the actual experimental first pulse (both highlighted in grey), showing that this population can be accounted for by dropoff.

Experiment	**2X Responder**	**Exp. First Pulse**	**Total Responses**	**Theoretical First Pulse**	**% N Pulsed First**
N25/25	0.784	0.256	1.04	0.282	78%
N8/8	0.747	0.194	0.941	0.206	63%
N25/25/24	0.794	0.309	1.103	0.303	79%

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