

Maternally Inherited Peptides Are Strain Specific Chemosignals That
Activate a New Candidate Class of Vomeronasal Chemosensory Receptor

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the University Program in Genetics and Genomics
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ABSTRACT

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Abstract

The chemical cues that provide an olfactory portrait of mammalian individuals are in part detected by chemosensory receptors in the vomeronasal organ (VNO). By and large, the pertinent receptor-cue combinations used for olfactory communication are unidentified. Here we identify members of the formyl peptide receptor (FPR) family of G protein coupled receptors as candidate chemosensory receptors in the VNO of mice. We demonstrate that N-formylated mitochondrially encoded peptides presented by the major histocompatibility complex (MHC) molecule H2-M3 stimulate a subset of the VNO sensory neurons (VSNs). We show that one VNO localized FPR, *Fpr-rs1*, is differentially activated by strain specific variants of N-formylated peptides. We show that N-formylated peptides can function as chemosignals in a strain selective pregnancy block. We propose that this link between self-recognition peptides of the immune system and chemosensory pathways provides a possible molecular means to communicate the nature of an individual's maternal lineage or strain.

Dedication

This work is dedicated to Summer, the most loving and supportive wife one could envision, and our beautiful daughter Esmé. None of this could have been possible without your love, support and encouragement for which I am eternally grateful.

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Acknowledgements

I would like to thank those educators who have been so patient with me: my high school science teacher Mr. Van Damm, Dr. Albert Matlin and Dr. David Benzing as advisors at Oberlin College, Dr. Bernard Strauss for my first lab position, Dr. Kathleen Millen for reminding me of the beauty and wonder of science, and Dr. Hiro Matsunami my fearless mentor whose kindness and intelligence are unsurpassed. I would also like to thank Hideto Kaba, Ron Yu, Limei Ma, Jie He and Ian Davison for all of their help on this project. Thank you Hubi Amrein, Dan Tracey, Minmin Luo and Joel Mainland for the many comments on my work. I would like to also thank Fan Wang, Doug Marchuk and Raphael Valdivia and Mike Ehlers for sharing their resources with me, Akemi Toyama, Rose Li and Wenling Liu for expert technical assistance and the members of the Matsunami lab for providing a great place to work.

This work was supported by grants from NIH, HFSP and DARPA.

1. Introduction

Animals garner a wealth of information from the olfactory signals emitted from other individuals. In rodents, both volatile molecules and non-volatile peptides excreted from conspecifics can provide information regarding the strain, sex, reproductive state, or genetic makeup of an individual through olfactory sensory neurons in the olfactory epithelium and VNO, a bone encapsulated, bilateral structure adjacent to the ventral, anterior nasal septum (Brennan and Zufall, 2006; Buck, 2000; Dulac and Torello, 2003; He et al., 2008; Luo et al., 2003). Though multiple families of G protein coupled receptors (GPCRs) expressed in the olfactory sensory neurons or in the VSNS have been characterized, the exact receptors and corresponding signals that govern the discriminatory abilities and behaviors of mice have proven difficult to identify (Buck and Axel, 1991; Dulac and Axel, 1995; Herrada and Dulac, 1997; Liberles and Buck, 2006; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Here I describe the identification of an uncharacterized class of chemosensory receptor in the VNO, a novel class of agonists, N-formylated peptides that are able to stimulate VSNS and one of these receptors, *fpr-rs1*. Finally, work shows that these N-formylated peptides are sufficient as cues of strain identity in a pregnancy block assay.

1.1 Olfactory Chemosensation

Chemosensation is the process whereby an organism receives chemical cues to assess the content and quality of its proximate environs. For mice the detection of chemicals is achieved through the taste, olfactory, and trigeminal systems. In a

biological context chemosensation is integral to foraging, predator-prey relationships, mate choice, the avoidance of noxious materials, and thus survival.

Olfaction can be divided into two distinct systems: the main olfactory system and the accessory olfactory system. Olfactory signaling is mediated by seven transmembrane spanning receptors, members of the G protein coupled receptor (GPCR) superfamily. GPCRs implicated in mammalian olfaction include odorant receptors (ORs) and trace amine associated receptors (TAARs) in the main olfactory epithelium, as well as the putative pheromone receptors (V1Rs and V2Rs) in the vomeronasal organ of the accessory olfactory system. Though it is known that mice utilize olfactory signals to discern characteristics of individuals, such as sex or group identity, the specific receptors and cues that initiate these cognitive processes remain elusive.

1.2 The Main Olfactory System

The main olfactory process is most often associated with the detection of volatile odors such as the scent of a rose or, more importantly for a mouse, the scent of a food source. Both pleasant and unpleasant odors are detected by the largest class of GPCRs, the odorant receptors (ORs) and a second class of GPCRs, the trace amine associated receptors (TAARs) (Buck and Axel, 1991; Liberles and Buck, 2006).

1.2.1 Odorant Receptors

The molecular characterization of the receptors responsible for the detection of chemicals was born in 1991 with the seminal work of Linda Buck and Richard Axel

(Buck and Axel, 1991). The ORs are members of the seven transmembrane spanning Class A (rhodopsin like) family of GPCRs, and the largest gene family in the mouse genome. There are two classes of ORs: the class I or fish-like receptors and a class II (Zhang and Firestein, 2002). It is thought that the class I receptors receive molecules that are hydrophilic and class II receptors are bound by hydrophobic odors. These receptors are found at the ciliary tip of olfactory sensory neurons in the main olfactory epithelium (Menco et al., 1997). Odorant binding to an OR activates the G protein, which in turn activates adenylate cyclase. This results in the conversion of ATP into cAMP, which then opens a cyclic nucleotide-gated ion channel, depolarizing the OSN and initiating an action potential that is transmitted to the MOB. Each neuron expresses only one allele of the thousands of possible OR alleles (Chess et al., 1994; Malnic et al., 1999; Vassar et al., 1993). For OSNs expressing the same OR the axons extend and converge to two (or one) glomeruli in each of the main olfactory bulbs (Mombaerts et al., 1996; Wang et al., 1998). The identity of the OR and the receptor derived cAMP levels are key components to the precise targeting of axons to the MOB (Imai et al., 2006; Wang et al., 1998).

1.2.2 Trace Amine Associated Receptors

TAARs are the most recent addition to the MOS. Work in Linda Buck's lab by Stephen Liberles identified the expression of these receptors in the MOE (Liberles and Buck, 2006). Like ORs these are in the class A (or Rhodopsin like) family of GPCRs and are expressed individually in neurons. Furthermore they were able to identify amines in urine that were either more abundant in males than females, or induced by stress, that were able to induce a calcium response in TAARs over-

expressed in a heterologous cell system. Similarly diluted urine also provided dose dependent responses.

As an aside, it was this work that was the impetus for us to question whether other unidentified GPCRs might be found in the VNO.

1.3 The Accessory Olfactory System

In most terrestrial vertebrates a secondary olfactory system exists, the accessory olfactory system (AOS). The accessory olfactory system is comprised of the VNO, AOB, and the higher brain centers that process the signals generated and transferred by these organs. Chemical signals from the environment are detected by sensory neurons in the VNO, translated into an electrical impulse that is transmitted to the AOB where signals are sorted and processed and passed to higher brain centers.

This system utilizes a different set of GPCRs in a distinct olfactory organ, the VNO. The wiring of the VSNs also differs in that the axons extend to AOB rather than the MOB. The segregation of the main and olfactory systems suggests that the functions of these two systems are also distinct. The exact function of the VNO is unclear. However, the accessory olfactory system appears to be associated with cues that effect aggressive, sexual and other behaviors by influence of the neuroendocrine system.

In addition to processes such as the finding of food and the avoidance of predators, rodents use the two olfactory systems in concert for communication (often through urinary cues) of the sex, status, and reproductive readiness of individuals.

1.3.1 The Vomeronasal Organ

The vomeronasal organ was first described by the Danish anatomist Ludvig Jacobson in 1813 (Jacobson et al., 1998). This elegant work described the anatomical structure of the organ in several species, detailing both the morphology and the innervation of this organ to what is now known as the accessory olfactory bulb.

“It is so carefully concealed that it has avoided discovery by the very discerning eyes of several anatomists... What in particular has hidden this organ from the eyes of the observers is a cartilaginous capsule that surrounds the parts comprising the organ, namely a secretory apparatus, a receptacle and an exit duct... This secretory organ is even more remarkable because it has its own large and specific nerves” (Jacobson et al., 1998).

This work is in itself “remarkable”. Not only had Jacobson discovered a unique organ in the midst of an era where comparative anatomy was the trend but also until recently little has expounded his original observations. However, as surgical, electrophysiological and genetic manipulations have developed, so has our rudimentary understanding of the accessory olfactory system.

1.3.1.1 Physiology

The VNO is a small, bone encapsulated, blind-ended, tube like structure in the nasal cavity. A pair of these structures lies bilaterally along the ventral anterior of the nasal septum. In the adult mouse this organ is approximately 3mm in length, and 700 μm across. Visually, a cross-section of the VNO can be dissected into neural and non-neural compartments. A crescent shaped neuroepithelium below a layer of non-neural supporting cells is found on the medial side of the mucus filled lumen of the VNO. The crescent of VSNs can bifurcated even further molecularly into an apical and basal layer. Molecularly the apical layer, which lies closest to the lumen, is

characterized by the expression of V1Rs, and the G protein subunit $G_{\alpha 12}$. The basal layer is comprised of neurons expressing V2Rs, an atypical class C V2R2, the g protein subunit $G_{\alpha o}$, and members of the non-classical MHC class 1b families H2-M1 and H2-M10. Vomeronasal sensory neurons, like OSNs, extend dendrites past a layer of supporting cells into the lumen. The dendritic tips, where the vomeronasal receptors are found, are terminated with up to 100 microvilli rather than the cilia found in the MOE (Ciges et al., 1977).

The lateral side is comprised of non-neural tissue including secretory glands and vasculature including a large blood vessel. In addition to providing blood and oxygen the arteriole is used to control the pumping mechanism of the VNO, which pulls chemical cues into the lumen. The stimulation of the nasalpalatine nerve is relayed by sympathetic fibers to the VNO, resulting in the constriction of the blood vessel, a consequential increase in the volume of the lumen, and thus the suction of cues into the blind-ended VNO. Nonsympathetic neurons drive the dilation of the blood vessel that results in a decrease of the luminal volume and consequently an expulsion of the contents within. It is interesting to note that stimulation of the nasalpalatine nerve also results in the dilation of the nasalpalatine duct, possibly allowing for a greater degree of interaction between the nasal and buccal cavities. The significance of this is unexplored.

1.3.1.2 The Vomeronasal Receptors

Vomeronasal receptors are comprised of two distinct families of GPCRs, that couple to two different G proteins, and express in two segregated regions. In the basal region neurons express a single V2R receptor family member as a heterodimer

with V2R2 and express the $G_{\alpha o}$ subunit (Berghard and Buck, 1996; Berghard et al., 1996; Herrada and Dulac, 1997; Martini et al., 2001; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). V2Rs are Class C GPCRs characterized by a long extracellular N-terminus. This long extracellular domain, though found in the T1Rs, is unique among olfactory chemoreceptors. In addition the H2-M10 family of MHC class Ib molecules is expressed in a large subset of the basal neurons (Ishii et al., 2003; Loconto et al., 2003). These MHCs are found only in a subset of basal VSNs and are proposed to serve a role as chaperones to the V2Rs (Ishii and Mombaerts, 2008).

V1Rs were first identified by Catherine Dulac in Richard Axel's lab (Dulac and Axel, 1995). V1Rs are found in the apical region of the VNO. These class A GPCRs – lacking the long N-terminus - are expressed with the $G_{\alpha i2}$ subunit.

Signaling for both receptor types, V1Rs and V2Rs is believed to be mediated by phospholipase C (PLC) pathway and TRPC2 (Dulac and Torello, 2003). Here ligand binding of VNO receptors activates PLC, perhaps through G proteins. In turn PLC hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). DAG then activates TRPC2 either directly or indirectly (Liman et al., 1999; Lucas et al., 2003; Spehr et al., 2002; Zufall et al., 2005). Several lines of evidence support this proposed mechanism. First, IP3 levels have reportedly increased following stimulation of VSNs (Krieger et al., 1999; Sasaki et al., 1999). Next, pharmacological inhibitor of PLC can block both calcium responses and action potentials in VSNs (Holy et al., 2000; Inamura et al., 1997; Spehr et al., 2002). Also, inhibitors of arachadonic acid synthesis, a downstream biproduct of DAG, have no effect of the urine evoked currents in VSNs (Lucas et al., 2003). Deletion of TRPC2 in mice results in several behavioral anomalies associated with VNO function. Both males and lactating females fail to display aggression in a

resident intruder assay (Kimchi et al., 2007; Leypold et al., 2002; Stowers et al., 2002). *Trpc2*^{-/-} mice display a significant increase in mounting behavior towards conspecifics of the same sex (Kimchi et al., 2007; Leypold et al., 2002; Stowers et al., 2002). Electrophysiological responses of VSNs to both diluted urine and sulfated steroids are also abolished in these animals (Holy et al., 2000; Nodari et al., 2008).

1.3.2 The Accessory Olfactory Bulb (AOB)

Axons from vomeronasal neurons are projected to the AOB with regionalized specificity. VSNs expressing V1Rs extend their axons to the anterior portion of the AOB and V2R expressing VSNs to the posterior (Belluscio et al., 1999; Rodriguez et al., 1999). The axons specified by a V1R or V2R are projected to multiple (6 – 30) glomeruli (smaller than those observed in the MOB), each of which is targeted by multiple receptors (Belluscio et al., 1999; Rodriguez et al., 1999; Wagner et al., 2006). In contrast to the precise targeting of ORs in MOB, the distribution of glomeruli can even vary between bulbs of an individual. Mitral cells in the AOB provide the first relay center of neural signals from the VNO. The dendrites of each mitral cell are also restricted to either the anterior or posterior halves of the AOB where they innervate multiple glomeruli (Del Punta et al., 2002b; Jia and Halpern, 1997; von Campenhausen et al., 1997). The activation of multiple glomeruli may then converge into fewer, or a single, output as second order mitral cells of the AOB tend to innervate glomeruli expressing the same V1R or V2R (Del Punta et al., 2002b). The dichotomy of the AOS is thus preserved in the output of the AOB, allowing for potentially separate functions for V1Rs and V2Rs. Axons of mitral cells extend down the accessory olfactory tract to the medial amygdala. Therefore signals

from the VNO are only a single synapse removed from affecting higher brain centers known to regulate behaviors including the aggressive and sexual behavior associated with the VNO. Mitral cells are connected by multiple periglomerular neurons that can provide inhibitory feed back to other mitral cells. This provides us with some insight into the signaling of the AOS but the method by which stimuli are processed in the AOB is far from resolved.

1.3.3 Developmental Origins in the Mouse

The origins of the VSNs are found on the 9th day of development in the anterior neural crest as the rostral neurogenic placode is formed (Cuschieri and Bannister, 1975a, b). Here, a layer of sensory cells is sandwiched between a superficial layer of nonsensory cells (epiblast) and the neural tube. It is from these olfactory placodes that the main olfactory and accessory olfactory systems are derived as the nervous layer proceeds to make the olfactory neurons and the nonneural layer develops into the supporting cells and Bowman's glands. On the 10th day the placode begins to deepen as it forms the olfactory pit. The AOS differentiates from the MOS on day 11 as a ventromedial invagination of the deepening olfactory pit (Cuschieri and Bannister, 1975a, b; Szabo and Mendoza, 1988). It is at this time that the dendrites of the developing VSNs appear at the epithelial surface and that the axons begin to connect with the nearby developing AOB. The tube like structure of the VNO is formed by day 12 from a fold of epithelium which initiates a caudal connection with the presumptive medial septum and then progressively fuses rostrally until only the anterior opening to the VNO remains (Cuschieri and Bannister, 1975b). Neurons continue to proliferate during development, particularly during the

first two weeks of postnatal life, extending axons to the AOB following a complex set of set of guidance cues, which have not yet been fully characterized.

Leutenizing hormone-releasing hormone (LHRH) and gonadotropin releasing hormone (GnRH) neurons found in the hypothalamus and forebrain (respectively) also find their origin in the medial end of the olfactory placode (Wray, 2002; Wray et al., 1989). It is tempting to speculate a functional significance related to the proximity of these two cell types with olfactory precursors. Clinical disorders such as Kallman's syndrome (or olfactory-genital dysplasia) do provide support for a developmental relationship but whether this relationship has any obligatory consequence to circuitry or olfactory regulation of hormonal levels is not clear.

1.3.4 Evolution

It is believed that evolutionary origin of VSNs predates the separation of teleosts and tetrapods (Grus and Zhang, 2009). The VNO is believed to have evolved as an olfactory organ of which specialization accompanies the vertebrate transition from aquatic environments to land. The evolutionary origins begin with a transformation and specialization of one type of chemoreceptor cell found in a general olfactory compartment of a common ancestor of all extant vertebrates (Bertmar, 1981). Several lines of evidence support this hypothesis. First, in some fish the olfactory epithelium is comprised of neurons that terminate with both cilia (as found in OSNs) and microvilli (as found in the VNO) (Hansen, 1994). Amphibians exhibit a segregation of these cell types as cells containing microvilli are found centralized in a cavity contiguous with the main olfactory compartment and mammals have a distinct nasal structure (Evans, 2003). In a sense the evolution of

the VNO mimics the development of the organ where the VNO develops as a specialization of olfactory cells, then segregates and eventually becomes separated as it is incased with cartilage and bone.

It is thought that V1Rs detect volatile airborne odorants and V2Rs detect non-volatile, soluble compounds (Dulac and Torello, 2003). Interestingly the vertebrate transition from water to land corresponded to a ~50 fold increase in the ratio of V1Rs (for volatiles) to V2Rs (for soluble compounds) further suggesting a specialized role for these receptor types (Shi and Zhang, 2007).

Most tetrapods have a secondary olfactory system with the notable exceptions of birds, old world monkeys and apes, including humans. Not only are the structures lost in these species but so have the functional genes encoding the molecular signaling pathways including the V1Rs, V2Rs, and Trpc2 (Liman and Innan, 2003; Shi and Zhang, 2007; Zhang and Webb, 2003). There are reports of a small number of V1Rs in humans (Rodriguez et al., 2000). However these are likely relics of incomplete pseudogenization of these receptors under relaxed selective pressure (Zhang and Webb, 2003).

1.3.5 Function

The function of the accessory olfactory system remains enigmatic and contentious. Originally posited as simply a secretory gland, or possibly a secondary olfactory or taste organ the current predominant view is that the VNO plays a role in mediating cues that illicit a variety of behaviors (Jacobson et al., 1998).

The evidence provided by genetic manipulations of the molecular machinery of the VNO suggests a role in both sexual and aggressive behaviors. Deletion of 16

V1Ra and V1Rb family members had a modest effect on male sexual behavior (Del Punta et al., 2002a). Males showed a decreased interest in females although they still showed a significant preference for females. No aggressive behavioral modifications were observed. A deletion of $G_{\alpha i2}$, the G-protein of the apical region of the VNO, resulted in a decrease in aggressive behavior and a lengthening of the latency preceding the initial attack (Norlin et al., 2003). Deletion of TrpC2 results in an increase in sexual and a decrease in aggressive behaviors (Kimchi et al., 2007; Leybold et al., 2002; Stowers et al., 2002)

The literature regarding the function of the AOS is often unclear or contradictory. However, four remarkable physiological responses have been, at least in part, attributed to processes of the AOS of mice.

1.3.5.1 The Lee-Boot Effect

One of the earliest demonstrations of a chemical cues influence on physiological outcomes of mice was observed in groups of females housed together. Andervont while studying the growth of hormone dependent tumors first described the earlier incidence, increased frequency and duration of estrous cycles when females were housed singly rather than in groups (and concurrently the development of the tumors was slowed) (Andervont, 1944). This work was furthered by the demonstration that groups of female mice, in the absence of males, became either pseudopregnant (a false state of diestrus where corpora lutea are not resorbed but remain excreting progesterone, thus mimicking a pregnant state) or in anestrous (noncycling and the corpora lutea absent)—a phenomenon often referred to as the Lee-Boot Effect (Dewar, 1959; Van Der Lee and Boot, 1955; Whitten, 1959). Others

have suggested that females in large groups are also pseudopregnant rather than the anestrus state suggested by Whitten, (Ryan and Schwartz, 1977). Regardless, a majority of females will return to estrus cycling if placed in a cage individually or the olfactory bulbs are removed (Mody, 1963; Van Der Lee and Boot, 1955; Whitten, 1970). Thus, a chemosignal excreted from females can inhibit or terminate the estrus cycle via an olfactory pathway. Though ablation of the olfactory bulb eliminates both the MOS and AOS it was later demonstrated by the surgical removal of the VNO that the AOS was central to the suppression of estrus (Ingersoll, 1980; Reynolds and Keverne, 1979; Vandenberg, 1983). Moreover, it was demonstrated that the urinary cue that elicited the suppression of estrus was not generated in females whose VNO had been removed (Lepri et al., 1985). The identity of the responsible urinary component and the mechanism by which it generates such a profound effect remains unknown, although some suggestions have been made. One group has suggested that 2,5-dimethylpyrazine is a necessary, but not sufficient, component of the suppressing cue but have not demonstrated this unequivocally (Jemiolo and Novotny, 1993; Ma et al., 1998). These works illustrate the complexity of studying vomeronasal signaling, where the VNO mediates the reception of a cue that induces the excretion of a cue that in turn suppresses the estrus cycle in others.

1.3.5.2 The Whitten Effect

The Whitten Effect stemmed from work on estrous delay in grouped females, when it was discovered that the estrous cycles of grouped female mice would be reinitiated when exposed to a male mouse (Whitten, 1956, 1959). When a male is introduced to a large group of females most of them, about 50% (much more than one would expect by chance), will have a release of gonadotropin from the anterior

pituitary resulting to a return to estrus cycling and hence mating on the third day following the introduction of the male (Bronson, 1974). The identity of the male cue or cues that induce this effect remain unknown, although it has been characterized as volatile, androgen and gonad dependent, found in urinary bladder, and to some extent species specific (it is absent in human male urine) (Bronson, 1974; Bronson and Whitten, 1968; Bruce, 1965; Whitten et al., 1968). Although this has been suggested to be a volatile cue, one study demonstrated that when the female could make contact with the male or his urine the incidence of estrus induction was almost twice that of females restricted from the odor source (Wilson et al., 1980). This may simply be a dosage effect or perhaps two components, a volatile and non-volatile component, function in concert.

The Whitten Effect is not generally characterized as a strain specific effect in mice but one study demonstrates that in contrast to most mice that mate on the third day, most BALB/cWt mice will mate on the first (Champlin et al., 1980). The frequency of mating on the first day is significantly increased if females are housed alone. Like most aspects of the Whitten Effect the cause of these observations remains undetermined.

The role of the VNO in the synchronization of estrus in females, by the presence of a male or his urine, has only been suggested in mice but has been demonstrated in rats; a rodent that is also susceptible to the Whitten Effect (Aron and Chateau, 1971; Chateau et al., 1976; Johns et al., 1978; Kaneko et al., 1980). Whitten attempted to verify a role of the VNO by lesioning the accessory olfactory nerve but never fully demonstrated this as his sham operated control females also failed to respond to the male cue. However, by occluding the VNO in female rats Johns *et al* were successful in demonstrating a role for the AOS in the detection of

male urine. Additionally, it was found that contact with the male urine was required for the urinary cue to be successful.

In summary it appears that the component of male urine responsible for the Whitten Effect is comprised of at least one volatile cue, and likely another nonvolatile compound, that is at least in part detected by the VNO.

1.3.5.3 The Vandenberg Effect

The acceleration of onset of puberty in female mice when raised with male mice is referred to as the Vandenberg Effect (Castro, 1967; Vandenberg, 1967, 1975, 1983; Vandenberg et al., 1975). The onset of puberty has been defined by the first estrus as determined by vaginal cornification and in other cases simply by uterine weight. The presence of a male can accelerate puberty onset in juvenile females from a few days to as much as 20 days. In contrast female mice (unless pregnant, lactating, or ovulating) actually delay the onset of puberty. The urinary cue derived from the male is androgen dependent and is equally effective if only 1 μ l, or a hundred-fold dilution is presented to the female (Drickamer, 1984; Vandenberg, 1983). A castrated male is ineffective at accelerating puberty (Bronson and Maruniak, 1975). However, if the castrated male is placed with the female in the presence of an intact male's urine the effect is restored. The nature of the cue also appears to be complex as urine alone is sufficient to induce an increase in uterine weight but contact with even a castrated male can double the effect (Bronson and Maruniak, 1975). Early efforts to identify the puberty accelerating cue or cues were unsuccessful. A fraction of urine containing peptides was found effective but further separation of the cues by high-performance liquid chromatography (HPLC) was unsuccessful (Vandenberg, 1983). More recently a number of groups have

suggested a number of chemicals and peptides that are able to induce the acceleration of puberty including: 5,5-dimethyl-2-ethyltetrahydrofuran-2-ol, 6-hydroxy-6-methyl-3-heptanone, 3,4-dehydro-exo-brevicommin, 2-sec-butyl-4,5-dihydrothiazole, α -farnesene, β -farnesene, and a hexapeptide derived from the N-terminus of a major urinary protein, EEARSM (Mucignat-Caretta et al., 1995; Nishimura et al., 1989; Novotny et al., 1999a; Novotny et al., 1999b). The abundance of cues suggested to regulate the Vandenberg effect may give one pause. It is possible that uterine weight, the measure by which all of these compounds have been identified, can be altered significantly indirectly as a result of toxicity. Indeed, toxicological screens for environmental endocrine-disrupting chemicals and estrogenic compounds utilize uterine weight as an indicator of toxicity (Padilla-Banks et al., 2001). On the other hand it may be a synergistic effect of both volatile and non-volatile estrogenic compounds in male urine that constitute the "responsible" cue.

The necessity of the VNO in detection of the estrus accelerating factor(s) was demonstrated by the interruption of the female AOS. Either removal of the VNO, a bilateral bulbectomy or a unilateral bulbectomy with a lesion of the contralateral vomeronasal nerve can eliminate the male's effect. In contrast, sham operated or unilaterally bulbectomized females are still responsive to the accelerating cue (Kaneko et al., 1980; Lomas and Keverne, 1982). In the context of potential inductive cues similar experiments will be necessary to verify that the acceleration of puberty resultant from urinary compounds is mediated by the AOS.

1.3.5.4 The Bruce Effect

One of the more remarkable, and more thoroughly characterized, physiological phenomenon attributed to the accessory olfactory system is termed the Bruce effect or pregnancy block. In 1959 while working with an outbred albino mouse strain termed P (for Parkes) and inbred CBA mice (substrain G), she observed that recently mated albino females when removed from the stud will terminate the pregnancy if the male is replaced 24 hours later by a male of a different, or "alien" strain (Bruce, 1959). Additionally, she observed that if the recently mated female was separated from the males by cage within the box of alien males the rate of termination was unchanged. The pregnancy was unaffected by the presence of the original stud, males of a similar strain, strange females, immature males or castrated males (Bruce, 1959, 1965). Thus in some way a female is able to "remember" the identity of the stud male and distinguish or respond differently to a dissimilar male.

The premise that the identity of the stud is remembered is supported by the observation that if the stud is removed 20 min following mating and exposed to the cage of the "alien" male instead, then the original stud can be used as the blocking agent (Rosser and Keverne, 1985). Moreover, the female must be exposed for a period of more than three hours to the soiled cage of the "alien" male for the full blocking effect of the stud to be realized. Others have found that if the female is removed and placed in a clean cage three hours after coitus that the female is then unresponsive to "alien" males (Lott and Hopwood, 1972). Thus two recognition events take place before a pregnancy block can occur: the stud's identity is established over a 3 to 4.5 hour mating period and then the second male is established as dissimilar to the stud at a time prior to implantation.

Bruce concludes that "contact" is not necessary because a female separated from males by a wire mesh cage also terminates the original pregnancy. However, Bruce in a more thorough description of these experiments describes the mesh cage as having a 1/3 inch mesh (Bruce, 1960b). Thus "contact" refers to sexual contact rather than any sort of tactile stimulus. She was also able to demonstrate that females placed in the soiled stock cages of five males (with cotton lab coat material as bedding) were also able to terminate pregnancies. By placing the female in the recently vacated cage of the five males every 12 hours for 3 days the presence of a male to induce a pregnancy block was unnecessary (Parkes and Bruce, 1962). Therefore unlike the inductive cues of the Whitten effect, where a single exposure to either a soiled cage or microliter volumes of urine is sufficient, the Bruce Effect requires a relatively intense exposure to a space previously inhabited by males (Bronson, 1971). Later the application of urine to oronasal groove became the method of choice for the induction of the Bruce effect (and other pheromonal studies) (Leinders-Zufall et al., 2004; Marchlewska-Koj, 1977; Wilson et al., 1980). Again contact with urine is necessary.

The parous female is susceptible to the alien males olfactory cue for only a limited amount of time (Bruce, 1960a; Bruce, 1961). Bruce found that females housed with a male for as few as 12hrs (the least amount of time reported) was sufficient for pregnancy block, most responsive during a 48 hour exposure and is most responsive during the first four days following coitus. By the seventh day of pregnancy the female will not terminate her pregnancy. Others found that three 15 minute exposures to an alien male daily for four days were sufficient to terminate pregnancy (Chipman et al., 1966). Bruce also found that a female could be repeatedly exposed to a pregnancy block for up to five months (the longest period of

time tested) without any consequence to her fertility (Bruce, 1962). The one peculiarity she did observe was a ten-fold increase in the rate of pseudopregnancies in the first estrous cycle following a pregnancy block. This may imply a long lasting effect on the pituitary axis of the neuroendocrine system.

Bruce also demonstrated that olfactory cues were utilized in this process as bulbectomized females were not responsive to the alien males aborting cue (Bruce and Parrott, 1960). Later, using zinc sulfate treatments to ablate the MOE and vomeronasal cauterization to ablate the VNO, it was demonstrated that damage to the female MOS reduced the time investigating male urine soaked bedding and that presence of the VNO, but not the MOE, was required for detection of the pregnancy blocking signal (Lloyd-Thomas and Keverne, 1982).

The efficacy of pregnancy block in different strains and species of mice has also been studied (Bronson and Eleftheriou, 1963; Bruce, 1968; Chapman, 1969; Chipman and Bronson, 1968; Godowicz, 1970; Marsden and Bronson, 1965). Marsden and Bronson demonstrated that C57BL/6 mice and 129/J were not influenced by the presence of "alien" males. Chipman and Bronson also found that C57BL/6J mice were not responsive to the blocking effects of a wild house mouse. Interestingly although Swiss ICR females were responsive to the inductive cue from wild mice the males were unable to block pregnancies themselves (Chipman and Bronson, 1968). Similar results have been found for the inbred KE strain in Poland (Godowicz, 1970). Bruce also observed different efficacies in the inductive abilities of males between inbred and outbred strains (Bruce, 1968). Together the evidence is clear that only males of some strains of mice are able to generate a pregnancy blocking cue. Likewise, for females, only certain strains are influenced by the terminating signal.

The phenomenon of the pregnancy block has only been described in members of the Muridae family of rodents. The effect has also been observed in deer mice, *Pseomyscus maniculatus bairdii*, voles, *Microtus pennsylvanicus*, *Microtus agestis* and *Microtus ochrogaster*, the northern collared lemming, *Dicrostonyx groenlandicus* and suggested in the bank vole *Clethrionomys glareolus* (Clarke et al., 1970; Clulow and Clarke, 1968; Clulow and Langford, 1971; Mallory and Brooks, 1980; Stehn, 1975). Notably absent from this list of rodents is the rat. Although the rat is responsive to the male excreted cues that shorten the estrous cycle of females the Bruce effect is not exhibited between two inbred rat strains: Long-Evans and Sprague-Dawley (Davis, 1969; Marchlewska-Koj, 1983).

Here I have provided a description of phenomenon mediated by the VNO. It should be emphasized however that behavior is certainly not an outcome mediated exclusively by the VNO. It seems that functionally a combination of cues received by both the MOS and AOS are utilized during chemosensory communication. The synergistic effect of the MOS and AOS on behavior is illustrated by the sexual behavior of male hamsters towards receptive females. It has been demonstrated rodents treated with a nasal lavage of zinc sulfate are anosmic due to damage of OSNs (Alberts and Galef, 1971). Neither an application of zinc sulfate, nor the severing of the vomeronasal nerve is sufficient to significantly alter male sexual behavior. However, if zinc sulfate treatment was administered following a vomeronasal nerve lesion male mounting behavior was eliminated (Meredith et al., 1980). Similarly, in the rat, a complimentary effect on the MOS and AOS is illustrated by the maternal role relegated to virgin females exposed to newborn pups. Typically the virgin female will avoid the unfamiliar pups for some period of time, if

not kill them. Modulation of olfactory pathways is sufficient to alter this avoidance behavior (Fleming and Rosenblatt, 1974a, b, c). The amount of time avoiding the pups is unchanged when the main olfactory bulb is damaged. In contrast, when the vomeronasal nerve is severed the latency period before the virgin female takes on the role of maternal provider is reduced. However, the reduction of this latency period is even further reduced when the two treatments are combined (Fleming et al., 1979).

1.4 Activators of Vomeronasal Chemosensory Receptors

The molecules that have been proposed to activate vomeronasal receptors fall broadly into two categories: volatiles and non-volatile peptides.

Table 1: Activators of VSNs

Volatiles		
Novotny's	pentyl acetate, 2-heptanone, 2,5-dimethylpyrazine, a and b farnesene, 6-hydroxy-6-methyl-3-heptanone, 2-sec-butyl-4,5-dihydrothiazole, 2,3-dehydro-exo-brevicommin	(Boschat et al., 2002; Leinders-Zufall et al., 2000; Ma et al., 1999; Novotny et al., 1990; Novotny et al., 1986; Novotny et al., 1999a)
Volatile odorants	hexadecanolide, muscone, patchone, durene, <i>p</i> -cresol, eucalyptol, isoborneol, borneol, fenchone, butyrophenone, methylanisole, myrtenal, phenafleur, dimethyl-3-octanol, helional, pentadecalactone and aubepine	(Sam et al., 2001)
	isobutylamine	(Del Punta et al., 2002a)
	ethyl acetate, ethyl propionate, ethyl vanilline, butanone	(Trinh and Storm, 2003)
	sulfated steroids	(Nodari et al., 2008)
Peptides		
MUP proteins	rMUP1	(Chamero et al., 2007)
MHC Class I a Peptides	AAPDNRETF, SYFPEITHI, SYIPSAEKI	(Leinders-Zufall et al., 2004)
ESP peptides	ESP1, 3, 4, 5, 6, 8, 15, 16, 18, 23, 24, 31, 34, 36 and 38	(Kimoto et al., 2005; Kimoto et al., 2007)
Bacterial associated peptides	fMLP, LipoxinA4, CRAMP, uPAR	(Riviere et al., 2009b)

1.4.1 Volatile Chemicals

1.4.1.1 Urinary Volatiles from Mice

Many of the chemicals that have been demonstrated to activate VSNs were first identified in the lab of Milos Novotny. Interested in the pheromonal responses of mice to urine he identified chemicals with the ability to inhibit or accelerate puberty, or promote the Whitten effect (Jemiolo et al., 1989; Jemiolo et al., 1986; Novotny et al., 1986; Novotny et al., 1999a; Novotny et al., 1999b). Later works were able to demonstrate the ability of these molecules to elicit responses in the VNO and even neurons expressing specific receptors (Boschat et al., 2002; Leinders-Zufall et al., 2000). Others found that mice lacking a cluster of V1Rs also were deficient in the response to two of these chemicals and isobutylamine (Del Punta et al., 2002a).

1.4.1.2 Odorants

Sam et. al have also been able to demonstrate that VSNs can be responsive to odorants (Sam et al., 2001). Eighteen odorants from a battery of 82 were able to produce a calcium response in dissociated neurons. The authors suggest that volatile cues may be used to instruct instinctive behaviors such as the avoidance of prey. Others have demonstrated that type 3 adenylyl cyclase (AC3) knockout mice, in which the main olfactory pathway is disrupted but the accessory olfactory system remains in tact, were still able to detect some volatile odorants in the VNO (Trinh and Storm, 2003). The detection of odorants in the VNO is perhaps not surprising though the roles for these volatiles are entirely unknown.

1.4.1.3 Sulfated Steroids

A tour de force from Tim Holy's lab identified molecules in the class of sulfated steroids as activators of the VNO (Nodari et al., 2008). They identified these compounds by repeated fractionation of female mouse urine and evaluating the responsive fractions ability to stimulate VSNs as determined by recording field potentials on a multi-electrode array. These molecules were isolated from BALB/c females, but not present in male urine, and quite interestingly could be found at higher levels in stressed mice. Additionally in TRPC2 $-/-$ mice the VSN response to both urine and sulfated steroids is abolished (Stowers et al., 2002). For the purpose of comparison they also examined the relative efficacies of all other previously proposed pheromones and found that sulfated steroids produced responses 30-fold greater than previously proposed VSN agonists. These molecules appear to activate neurons in the apical region of the VNO, but the corresponding receptors are unknown.

1.4.2 Non-volatile Peptides

1.4.2.1 Major Urinary Proteins

As the name suggests, Major Urinary Proteins (or MUPs) are the predominant class of proteins found in mouse urine. These proteins are of particular interest because expression of these proteins has been demonstrated to be both sex and strain specific. The first identified class of peptide agonists of a physiological response associated with the vomeronasal system was the major urinary proteins (Mucignat-Caretta et al., 1995). This work suggested that a hexapeptide (EEARSM) was sufficient to accelerate the onset of puberty in female mice. More recently work

has demonstrated that a recombinant MUP1 protein could both stimulate V2R expressing VSNs and promote male-male aggression in mice (Chamero et al., 2007). This work directly demonstrates activation of VSNs. In contrast the activity of EEARSM is only implied, and indeed others have been unable to detect any vomeronasal activity generated by this peptide (Nodari et al., 2008).

1.4.2.2 ESPs

Kazushige Touhara's group made the first pairing of a peptide ligand and a specific family of vomeronasal receptors, the V2Rps (Kimoto et al., 2005). In this work they found that bedding soiled by males activated a small number of neurons in the VNO as determined by an antibody to c-fos, a member of the immediate early gene family of transcription factors expressed upon activation of a neuron. Most of these activated neurons expressed members of the V2Rp family of receptors. They were then able to identify extracts from the extraorbital lachrymal gland, and more specifically a peptide ESP1, to be responsible for a majority of the activity observed in neurons expressing V2Rps. Moreover they were able to identify this peptide in the tears of mice. Further work elaborated upon the glandular, strain, sex, age, and species specific expression of ESPs and demonstrated their ability to stimulate VSNs but not OSNs (Kimoto et al., 2007). It is proposed that these peptides are mediated by contact. By the nature of their differential expression between ages, strains and sexes of mice this family of 38 peptides is sufficient to specify many aspects of an individual. The behavioral or physiological outcomes associated with detection of these peptides are not yet determined.

1.4.2.3 MHC Associated Peptides

The identity cues describing strain have in part been attributed to differences in MHC composition. Interestingly, MHC class 1 molecules, typically associated with the immune system, are implicated in both the presentation and detection of VNO chemosensory cues.

In the immune system MHC class 1 glycoproteins are found at the cell surface of most nucleated cells bound to β 2-microglobulin. The MHC molecule is loaded with an intracellular derived peptide to its binding cleft, bound to β 2-microglobulin then transported to the cell surface. Together the MHC molecule, β 2-microglobulin, and the peptide form an antigen that is recognized by receptors surveilling T cells. The MHC molecules are the most polymorphic proteins between individuals. The great variety is offers an individual the ability to recognize a vast array of peptides from invading bacteria. As a consequence individuals have unique sets of peptides presented by polymorphic MHC class I molecules and consequently a distinctive chemical signature. Genetic variations within the H2 locus including MHC class 1 genes are known to influence urine odor, mate choice, and the maternal recognition of pups (Yamaguchi et al., 1981; Yamazaki et al., 2000; Yamazaki et al., 1988) Moreover, antigenic peptides presented by MHC class 1 molecules are known to stimulate VSNs and elicit physiological effects (Leinders-Zufall et al., 2004).

Much of the work on MHC associated peptides as chemosensory signals originates with the observation that female mice were found to have a preference for males with more dissimilar genotypes at the MHC locus (Yamazaki et al., 1976). A greater diversity of MHC genes would offer more resilience to bacterial infection and thus result in greater fitness of an individual. How could one mouse be discriminated from another? It was determined that mice could discriminate between the urinary

odors of mice that varied only at the MHC locus (Yamazaki et al., 1983a; Yamazaki et al., 1986; Yamazaki et al., 1983b; Yamazaki et al., 1976; Yamazaki et al., 1979). Moreover, they discovered that genetic differences in the MHC, and more specifically H2-K, could explain the strain specific effects of the pregnancy block (Yamazaki et al., 1986; Yamazaki et al., 1983b). Later others supported these findings by demonstrating that peptides presented by H2-K could stimulate VSNs (Leinders-Zufall et al., 2004). Additionally when peptides were pooled, in order to mimic the strain specific expression of H2-K peptides, the blocking of pregnancy was observed.

In addition to the classical MHC class 1 molecules, the H2 locus of mice encodes 3 families of non-polymorphic MHC class 1b molecules. H2-M3 is one well characterized member of this class of molecules. H2-M3 was originally identified through cytotoxic T lymphocyte assays as a component of the maternally inherited antigen (Wang et al., 1991, 2002). This MHC molecule, expressed ubiquitously in mice, presents two strain specific self-recognition peptides, ND1 and CO1, encoded in the mitochondrial genome (Loveland et al., 1990; Morse et al., 1996; Wang et al., 1995). ND1 is derived from the nine N-terminal amino acids of NADH dehydrogenase 1: MFFINXLTL. The identity of the sixth amino acid varies between strains and is either A, I, T, or V (Table 2 Table 2: N-terminal Polymorphisms in Mouse Mitochondrial Proteins). Similarly, CO1 is an N-terminal derivative of Cytochrome C Oxidase 1 (MFXNRWLFS where the third position is I or T).

Table 2: N-terminal Polymorphisms in Mouse Mitochondrial Proteins

Mouse Strain	ND1	CO1	ND2
C57BL/6J, CBA	f-MFFIN I LTL	f-MF I NRWLFS	f-MNPITL A II
BALB/cJ	f-MFFIN T LTL	f-MF I NRWLFS	f-MNPITL A II
NZB/B1NJ	f-MFFIN A LTL	f-MF I NRWLFS	f-MNPITL T II
LP/J	f-MFFIN I LTL	f-MF T NRWLFS	
MilP, WLA76, BFM	f-MFFIN V LTL		

As a consequence of being derived from mitochondria, the genes encoding ND1 and CO1 are maternally inherited. Thus, mice with mothers of the same strain will share identical ND1 and CO1 peptides. Both peptides are formylated at the N-terminal methionine, a protein modification characteristic of prokaryotes and organelles originating as prokaryotic endosymbionts such as mitochondria.

In a screen for uncharacterized chemosensory GPCRs in the VNO, we identified members of formyl peptide receptors, the FPRs, as a candidate class of chemosensory receptor. The first characterized members of the FPR family, Fpr1 and Fpr-rs2, are not found in the VNO but are receptors implicated in the immune response of leukocytes. These chemotactic receptors target neutrophils and monocytes to N-formylated peptides at sites of bacterial infection or cellular damage where N-formylated peptides are released (Boulay et al., 1990; Carp, 1982; Le et al., 2002; Schiffmann et al., 1975). We find that one VNO specific FPR, Fpr-rs1, is differentially activated by the maternally inherited, strain specific, N-formylated peptides presented by H2-M3 but not other agonists known to stimulate Fpr1 or Fpr-rs2. Additionally, we show that N-formylated peptides can both stimulate the VNO and elicit strain specific physiological effects. Together, these results describe novel

chemosensory signals and receptors as well as provide the first link between an individuality cue of the MHC and a defined chemosensory receptor.

Recently, others have independently identified FPRs as a candidate class of vomeronasal receptors activated by peptides and other molecules associated with pathogens or pathogenic status (Riviere et al., 2009a).

2. Identification of a Candidate Class of Chemosensory Receptor

2.1 An In Situ Screen for Novel Chemosensory GPCRs

The olfactory and vomeronasal GPCR family repertoire is significantly larger in the rodent species such as mouse and rat than in the primates such as human, chimpanzee and rhesus macaque, likely reflecting the relative importance of chemical cues in each lineage (Young et al., 2002). To identify previously uncharacterized mammalian chemosensory receptors we designed a screening strategy that selected for proteins that: 1) are GPCRs, 2) are not known chemosensory receptors, 3) belong to a receptor family that is larger in mice than in humans, and 4) are expressed in a discrete subset of olfactory or vomeronasal neurons. A comparison of human and mouse GPCRs from the GPCRDB information system excluding the odorant receptors (ORs), the trace amine associated receptors (TAARs), the V1Rs and the V2Rs identified 32 mouse genes without obvious human orthologues (Table 2) (www.gpcr.org/7tm/, (Horn et al., 2003)).

Table2: Screen for Novel Chemosensory GPCRs

	GPCRDB	Description/Gene	Genbank Accession #
1	Q921A8_MOUSE	Putative G-protein coupled receptor/ Csprs	AJ401359
2	Q3U0D6_MOUSE	Putative G-protein coupled receptor homolog/ Csprs	AK156961
3	Q8K3M8_MOUSE	Melanin-concentrating hormone receptor 1 alternate form/ Gpr24	AY049011
4	Q3KNA1_MOUSE	MAS-related GPR, member B2/ Mrgprb2	BC107391
5	Q4U2P4_MOUSE	Mu opioid receptor isoform MOR-1N/ Oprm1	AF260311
6	Q4VA00_MOUSE	G protein-coupled receptor 103/ Gpr103	BC096610 AK034431
7	Q3UH90_MOUSE	latrophilin 1/ Lphn1	AK147519
8	Q543S8_MOUSE	chemokine (C-C) receptor 2/ Ccr2	AK138599
9	Q71MR8_MOUSE	Formyl peptide receptor-related sequence 6/ Fpr- rs6	AF437512
10	Q71MR7_MOUSE	Formyl peptide receptor-related sequence 7/ Fpr- rs7	AF437513
11	O88538_MOUSE	N-formylpeptide receptor-like 4/ Fpr-rs4	AF071182
12	Q8CH74_MOUSE	Mu opioid receptor variant Q/ Oprm1	AF346813
13	Q3UG61_MOUSE	G protein-coupled receptor, full insert/ Mrgprb1	AK148108 AK148114 AK148044 AK148273
14	Q3UFW7_MOUSE	G protein-coupled receptor, full insert/ Mrgprb1	AK148279 AK148256 AK148074
15	Q2VY20_MOUSE	Orphan G protein-coupled receptor TRHRL2/ Gpr139	AY485344
16	Q3UG50_MOUSE	similar to G protein-coupled receptor/ Mrgprb10; G370024M05Rik	AK148121
17	Q3UZR6_MOUSE	G protein-coupled receptor 61/Gpr61	AK133702
18	Q8BLT7_MOUSE	hypothetical Rhodopsin-like GPCR superfamily containing protein/ A530099J19Rik	AK041317
19	Q7TN51_MOUSE	MRGB8/ Mrgprb8;MrgB8	AY266419

Table 3: continued

20	Q544V2_MOUSE	LYSOPHOSPHATIDIC ACID RECEPTOR (EDG-2)/ Edg2	AK030330
21	Q4VA66_MOUSE	Super conserved receptor expressed in brain 3/ Gpr173	BC096520
22	Q810W6_MOUSE	CXC chemokine receptor 1/ Il8ra	AY390263 AY749637
23	Q91ZC1_MOUSE	G protein-coupled receptor/ Mrgprb3;MrgB3	AY042201
24	Q3ZAW3_MOUSE	Neuropeptide Y receptor Y6/ Npy6r	BC103621 BC103622 BC103667 BC103620
25	Q3U1A9_MOUSE	MAS-related GPR, member A2/ Mrgpra2	AK156116
26	Q91ZC0_MOUSE	G protein-coupled receptor/ Mrgprb4;MrgB4	AY042202
27	Q8BY68_MOUSE	similar to PUTATIVE PURINERGIC RECEPTOR P2Y10/ A630033H20Rik; P2ry10	AK041740
28	Q91ZB9_MOUSE	G protein-coupled receptor/ Mrgprb5;MrgB5	AY042203
29	Q6YC50_MOUSE	Mu opioid receptor variant CII/ Oprm1;Oprm	AY160190
30	Q548Z8_MOUSE	Blue opsin/ Opn1sw	AF190670
31	Q543T0_MOUSE	SOMATOSTATIN RECEPTOR TYPE 1/ Sstr1	AK046464
32	Q498A2_MOUSE	MAS-related GPR, member G/ Mrgprg	BC100302

We assessed the mRNA expression of these 32 receptors on coronal sections of the MOE and the VNO by *in situ* hybridization. The results revealed expression of five FPR family members, including *fpr-rs1*, *-rs3*, *-rs4*, *-rs6* and *-rs7*, restricted to a subset of VSNs.

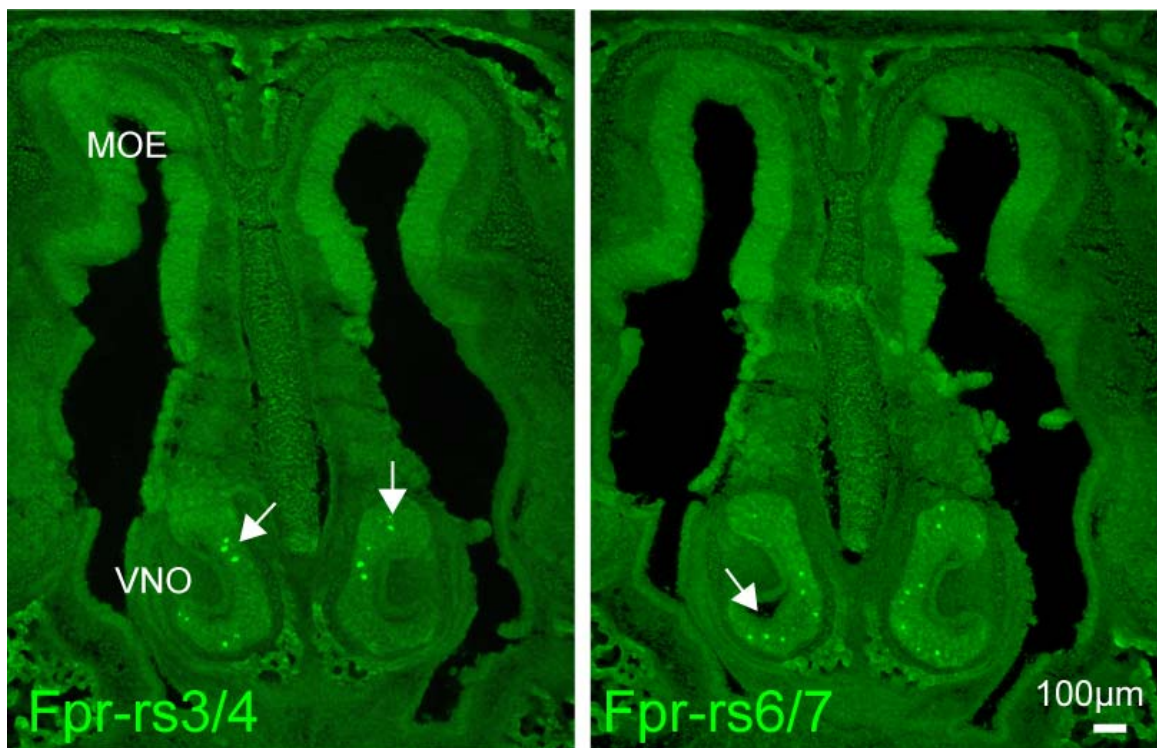


Figure 1: *In Situ* Screen Result

A representative result of an *in situ* hybridization screen of sensory tissues in coronal sections of the mouse anterior for putative chemosensory receptors. The results revealed that *Fpr-rs3* and *4*, and *Fpr-rs6* and *7* are expressed in the VNO but not the MOE. Similar results were found for *Fpr-rs1* (data not shown).

2.2 Genomic Analysis of Formyl Peptide Receptors

To examine the composition of the mouse FPR family and to assess whether similar receptors may be present in other species we conducted a genomic and phylogenetic analysis of these genes from divergent species. In the mouse genome, the single exon genes encoding the seven FPRs, Fpr1, Fpr-rs1, -rs2, -rs3, -rs4, -rs6, and -rs7, are situated over 5 Mbps on chromosome 17. Curiously, the FPRs are located amongst a grouping of known vomeronasal chemosensory receptors, V1Rs and V2Rs (Figure 2).

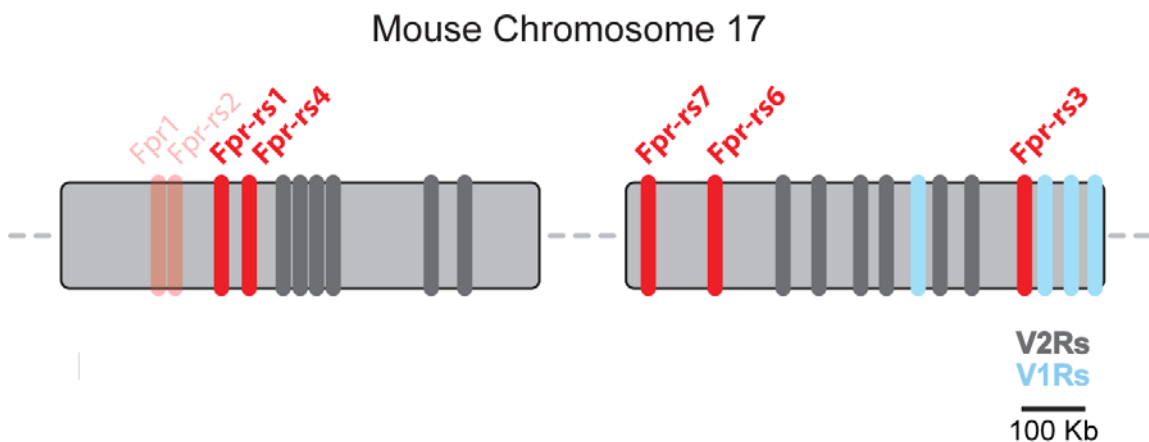


Figure 2: Genomic Organization of FPRs

A graphical representation of two .75 Mb regions on mouse Chromosome 17 shows that FPR genes (red) are encoded in two clusters among putative pheromone receptors, V1Rs and V2Rs (light blue and dark grey respectively). The first cluster of FPRs begins at the 18th Mb of Chromosome 17; the second cluster begins at Mb 20.2. Fprs found in the VNO are in bright red. Other family members not found in the VNO are in faded red.

A search of homologous genes in numerous genome sequences revealed that the composition of FPR families differs amongst mammalian species. For example,

seven family members are found in mice, six in rats, three in primates, but only one in dogs. No orthologous receptors were found in non-mammalian species including zebrafish, frog, and chicken. In species with three or fewer family members the receptors were always found in a single genomic cluster, whereas mice and rats have two gene clusters, suggesting a recent duplication. A phylogenetic tree generated using the neighbor-joining method showed that FPRs may be divided into 5 monophyletic clades (Figure 3) (Tamura et al., 2007). The Fpr1 clade is congruous to the widely accepted mammalian phylogeny, indicating that these sequences are truly orthologous. In contrast, three clades demonstrate lineage-specific expansions of FPRs, represented by platypus and opossum species, primates, and rodents. Mouse FPRs are 51-95% identical to one another, only 34-37% identical to the most similar non-family member, chemokine like receptor 1 (cmklr1), and less than 20% identical to known chemosensory receptors (Table 3 and Table 4). These results show that some FPR family members, much like other chemosensory receptors, are undergoing rapid evolution.

Table 3: Percent Amino Acid Identity between FPRs

	Fpr1	Fpr-rs1	Fpr-rs2	Fpr-rs3	Fpr-rs4	Fpr-rs6	Fpr-rs7
Fpr1							
Fpr-rs1	58						
Fpr-rs2	61	81					
Fpr-rs3	55	65	66				
Fpr-rs4	55	66	68	81			
Fpr-rs6	52	59	62	75	73		
Fpr-rs7	51	61	61	74	72	95	
Cmklr1	37	35	37	37	34	36	36

Table 4: Percent Amino Acid Similarity between FPRs

	Fpr1	Fpr-rs1	Fpr-rs2	Fpr-rs3	Fpr-rs4	Fpr-rs6	Fpr-rs7
Fpr1							
Fpr-rs1	73						
Fpr-rs2	76	88					
Fpr-rs3	70	76	78				
Fpr-rs4	69	79	79	91			
Fpr-rs6	62	74	75	86	83		
Fpr-rs7	67	74	75	85	83	97	
Cmklr1	54	56	56	56	55	57	56

2.3 Expression Analysis of Formyl Peptide Receptors

2.3.1 Analysis within different tissue types

To assess the tissue-specific expression of FPRs in the mouse we next performed RT-PCR with sequence specific primers on 14 distinct cDNA samples generated from various sensory and non-sensory tissue samples (Figure 4).

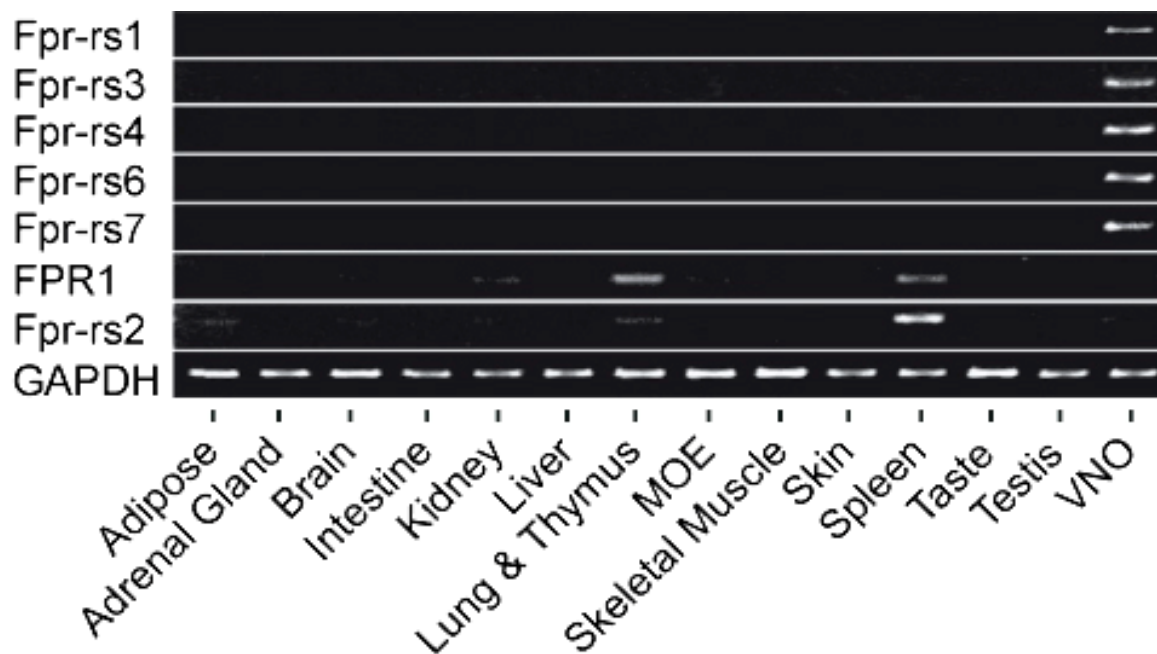


Figure 4: RT-PCR

Reverse Transcriptase PCR of FPR family members using sequence specific primers. Fpr-rs1, Fpr-rs3, Fpr-rs4, Fpr-rs6 and Fpr-rs7 are specifically expressed in the VNO.

Consistent with previous reports we found that Fpr1 and Fpr-rs2, two family members with an established role in immune response pathways, were expressed by spleen and lung/thymus (Gao et al., 1998). In contrast, expression of the five other FPRs - Fpr-rs1, -rs3, 4, 6, and 7 - is restricted to the VNO. The absence of expressed

sequence tags in the National Center for Biotechnology Information (NCBI) database as well as comprehensive analysis of GPCR expression by real-time RT-PCR also supports the notion that transcription of these genes is not widespread (Chamero et al., 2007; Dulac and Axel, 1995; Herrada and Dulac, 1997; Martini et al., 2001; Matsunami and Buck, 1997; Regard et al., 2008; Rodriguez et al., 1999; Ryba and Tirindelli, 1997; Silvotti et al., 2007).

2.3.2 Analysis within the VNO

We next characterized expression of FPRs within the VNO by *in situ* hybridization. Consistent with RT-PCR results, this analysis confirmed the expression of Fpr-rs1, Fpr-rs3 and 4, as well as Fpr-rs6 and 7 in a subset of the VSNs. We found that FPRs were expressed in ~2.7% (43/1580) of VSNs (Figure 5).

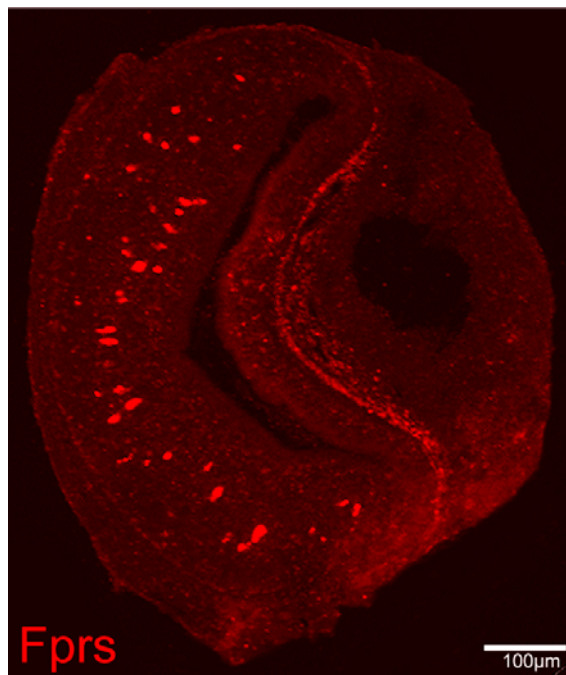


Figure 5: In Situ Hybridization of FPRs

This is a representative image of an *in situ* hybridization using pooled probes of FPR family members on a section of a VNO.

No Fpr1 or Fpr-rs2 expression was observed in the VNO. We found no obvious difference in expression between neonates and adult mice, or between males and females (data not shown).

2.3.2.1 FPR expression is Predominantly localized to the Apical Layer

VNO sensory epithelium is divided to two layers, apical and basal, which are distinguished from one another by the differential expression of the receptors, the V1Rs and the V2Rs, and the G proteins $G_{\alpha i2}$ and $G_{\alpha o}$. By using two-color *in situ* hybridization we determined whether transcription was constrained to the distinct populations of apical or basal neurons. To distinguish the apical and basal layer of the VNO we used probes specific for $G_{\alpha i2}$, expressed in apical neurons, and V2R2, a non-conventional V2R member that is widely expressed in the basal layer, as markers (Berghard and Buck, 1996; Martini et al., 2001; Ryba and Tirindelli, 1997). Using a mix of FPR probes, we observed that 88% of the FPR-expressing neurons are co-expressed with $G_{\alpha i2}$, and 99% segregated from V2R2 (Figure 6). Further investigation into the percentage of neurons that appeared outside of the $G_{\alpha i2}$ expressing neurons demonstrated that Fpr-rs1 is expressed in both the apical and basal layers, though predominantly in the basal zone.

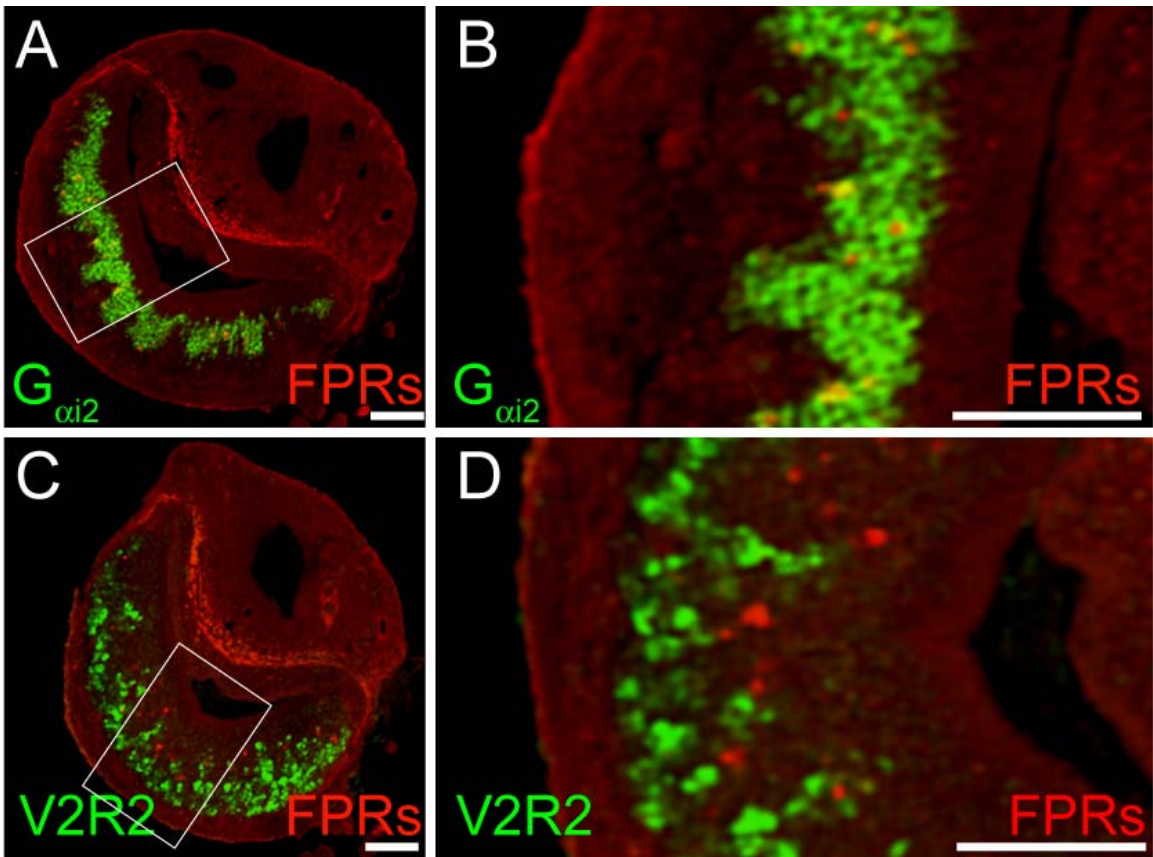


Figure 6: Most Fprs Express in the Apical Layer of the VNO

A) In situ hybridization demonstrates that most Fprs are co-localized with Gai2 expressing neurons and C) segregated from V2R2 expressing neurons. B) and D) are magnified images of the regions specified by boxes in A) and C) respectively.

2.3.2.2 FPRs are not co-expressed

Within the VNO V2Rs are co-expressed with a member of the V2R2 sub-family while only one V1R is thought to be expressed in a given neuron (Chamero et al., 2007; Dulac and Axel, 1995; Herrada and Dulac, 1997; Martini et al., 2001; Matsunami and Buck, 1997; Rodriguez et al., 1999; Ryba and Tirindelli, 1997; Silvotti et al., 2007). We asked whether the FPR receptors are co-expressed with each other. Using probes corresponding to the open reading frames we cannot distinguish the expression of Fpr-rs3 from Fpr-rs4, or Fpr-rs6 from Fpr-rs7 as high

identities in nucleotide coding sequences (Figure 7 A, B, C). Therefore we used 3' untranslated region probes to discriminate these FPRs. Our results suggest that different FPRs are expressed by different neurons, consistent with results by Riviere *et al* (Figure 7 D, E) (Riviere et al., 2009b). Similar expression patterns were obtained with both C57BL/6J and BALB/cJ mouse strains (data not shown).

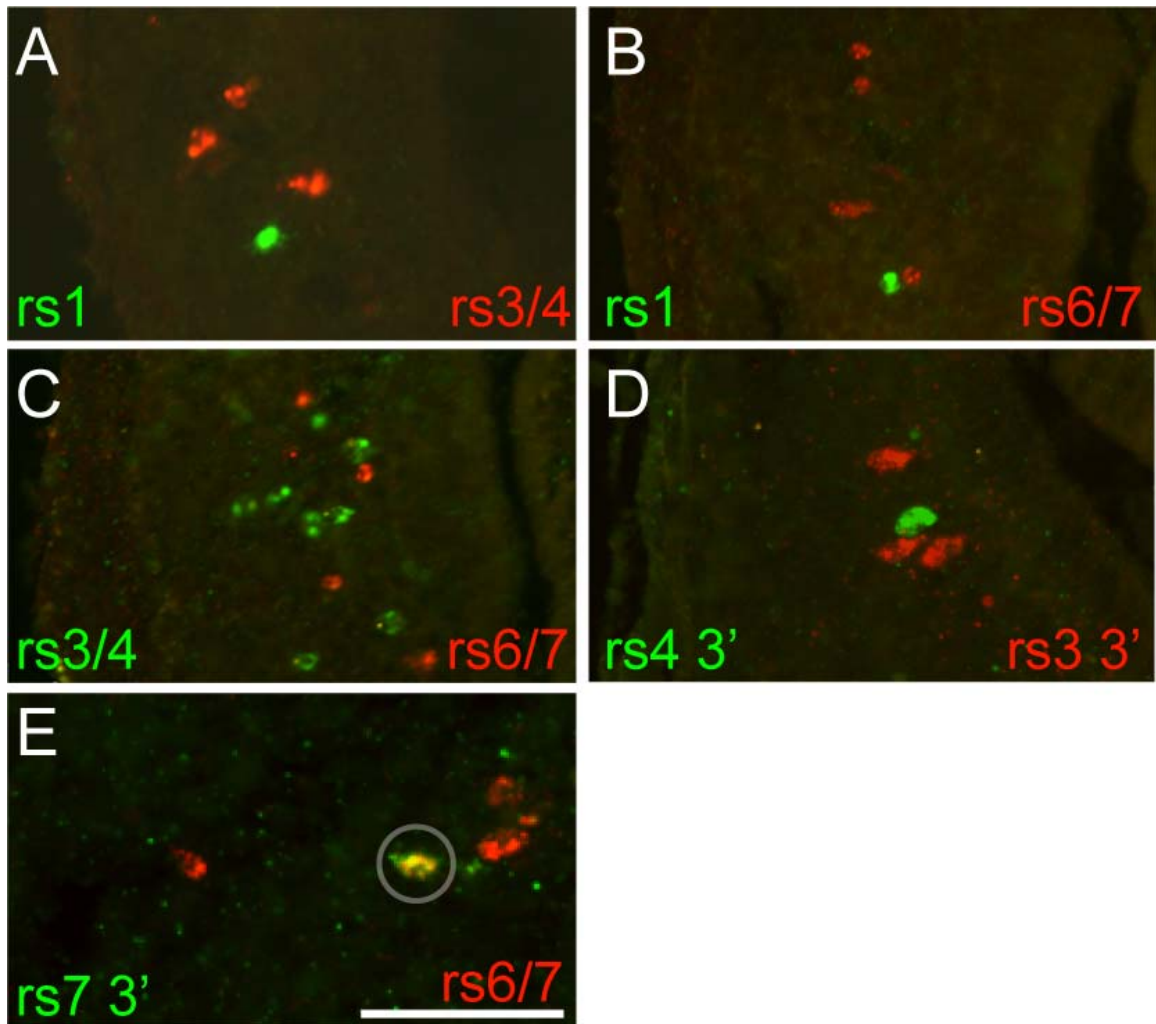


Figure 7: Fprs are not expressed with one another

A) Full length probes to fpr-rs1 and a probe that binds both fpr-rs3 and 4 do not co-localize. B) Full length probes to fpr-rs1 and a probe that binds both fpr-rs6 and 7 do not co-localize. C) Probes that bind both fpr-rs3 and 4 do not co-localize with probes that bind to both fpr-rs6 and rs7. D) Probes to the 3' UTR of fpr-rs3 and fpr-rs4 do not co-localize. E) Probes specific to the 3' UTR of fpr-rs7 only label a subset of the population of cells that are labeled with a probe that binds both fpr-rs6 and rs7.

2.3.2.3 FPRs are not expressed with V1Rs

We then asked whether these receptors are co-expressed with the V1Rs that are expressed in the apical domain of the VNO. To do so, we cloned a diverse set of 75 V1Rs covering ~40% of all V1R family members, and pooled full-length clones from each V1R sub-family to generate probes (Rodriguez et al., 2002). We found that FPRs and V1Rs were expressed by distinct populations of VSNs (Figure 8). Consistent with the characteristic expression patterns of other known chemosensory receptors, this data suggests that FPRs are expressed individually in a subset of VSNs.

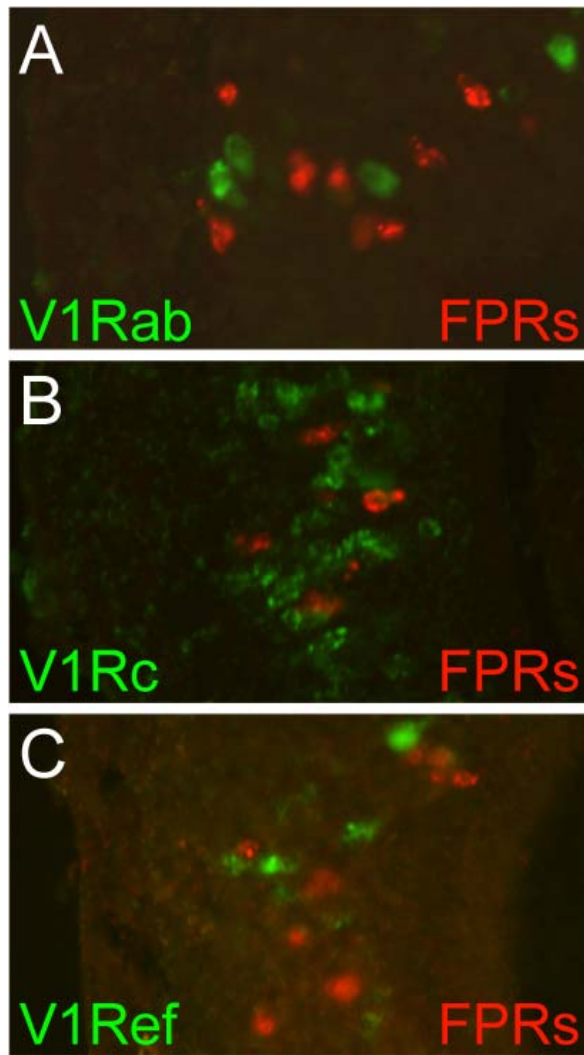


Figure 8: FPRs are not expressed with V1R family members

Pooled full-length probes to FPRs did not co-localize to cells expressing pooled probes to A) V1Ra and b family members B) V1Rc family members or C) V1Ref family members. Similar results were found for all other V1R families (data not shown).

3. N-formylated Peptides Stimulate the VNO

What might be the function of FPRs in the VNO? We hypothesized that FPR family members expressed in the VNO may be activated by N-formylated peptides, cognate ligands of Fpr1. Moreover, if FPR family members function as chemical detectors in the VNO they may be activated by chemicals that are relevant to VNO function. Two polymorphic N-formyl peptides encoded by mitochondrial genome are presented by H2-M3, an MHC class 1b antigen presenting molecule (Loveland et al., 1990; Morse et al., 1996; Wang et al., 1991). Variants of these nine amino acid peptides differ between mouse strains and have been demonstrated to be self-recognition molecules in cytotoxic T lymphocyte response and tissue graft rejection assays (Chan and Fischer Lindahl, 1985; Fischer Lindahl et al., 1980). We hypothesized that FPR family members expressed in the VNO might respond to the polymorphic mitochondrially-encoded peptides, potentially serving a function in individual recognition.

3.1 Field Potential Recordings

First, to assess the ability of a mitochondrially-encoded N-formylated peptide to stimulate the VNO, we performed field potential recordings from the intact sensory epithelium of the VNO (Figure 9). We examined the response of the VNO to ND1-6A. We found that VSNs respond to ND1-6A in a significant, dose dependent manner, with a detection threshold near 10^{-11} M ($F(1,33) = 172, p < 0.0001$). This highly sensitive response is comparable to previously described detection thresholds of small molecules and peptides that stimulate the VNO (Chamero et al., 2007; Kimoto

et al., 2005; Kimoto et al., 2007; Leinders-Zufall et al., 2004; Leinders-Zufall et al., 2000).

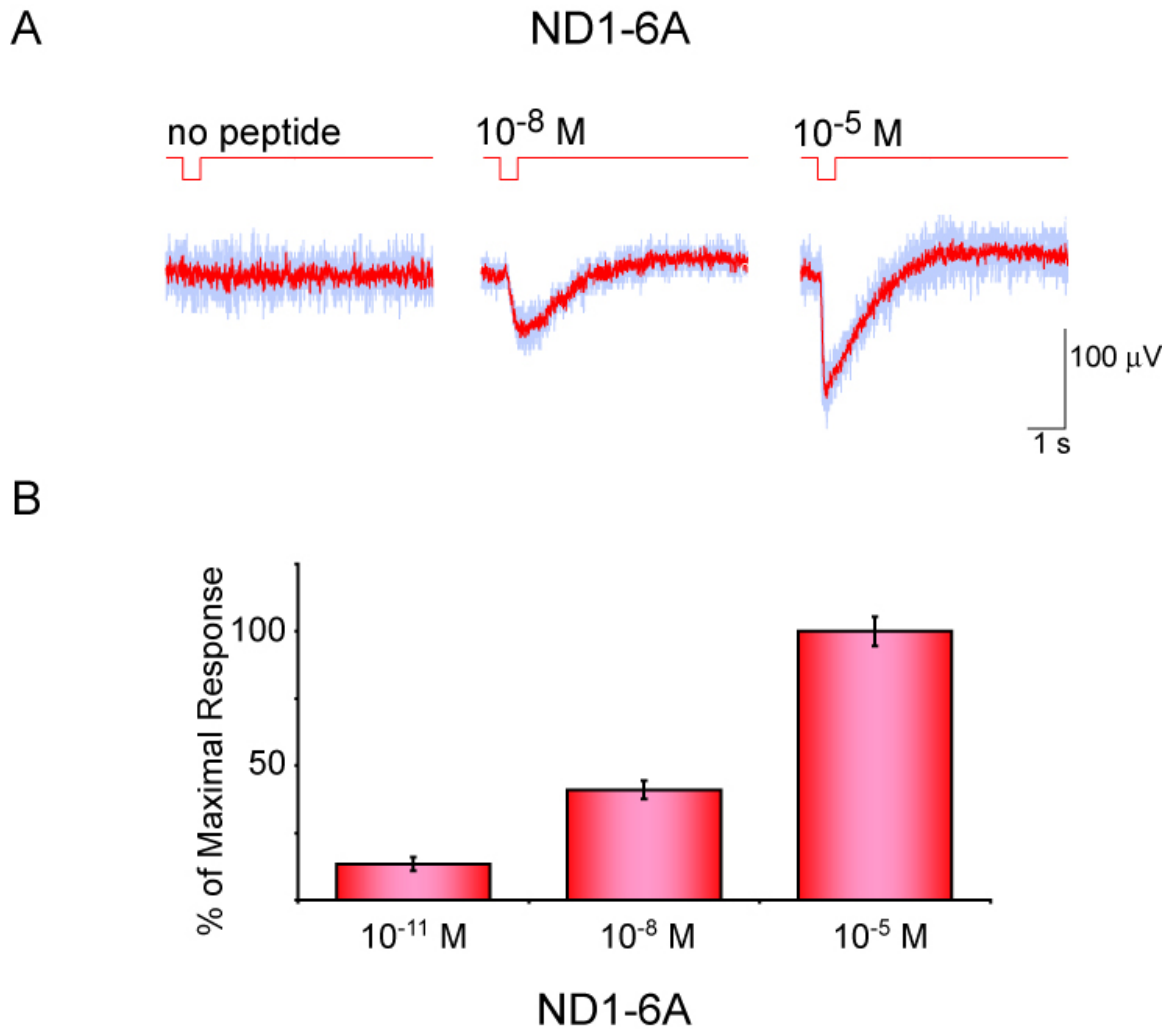


Figure 9: Field Potential Recordings

A) Dose dependent responses are observed to the ND1-6A peptide. B) A graphical representation of the responses

3.2 Calcium Imaging of Dissociated VSNs

Next, to observe N-formylated peptides stimulation of the VNO at a cellular level, we performed ratiometric calcium imaging on dissociated VSNs from C57BL/6J and BALB/cJ mouse strains. We found that a small subset of KCl responding C57BL/6J neurons (5/521) responded to the N-formylated peptides (Figure 10 A-C). Similarly, we found that a small percentage (16/1665) of BALB/cJ neurons differentially responded to individual N-formylated peptides (Figure 10 E).

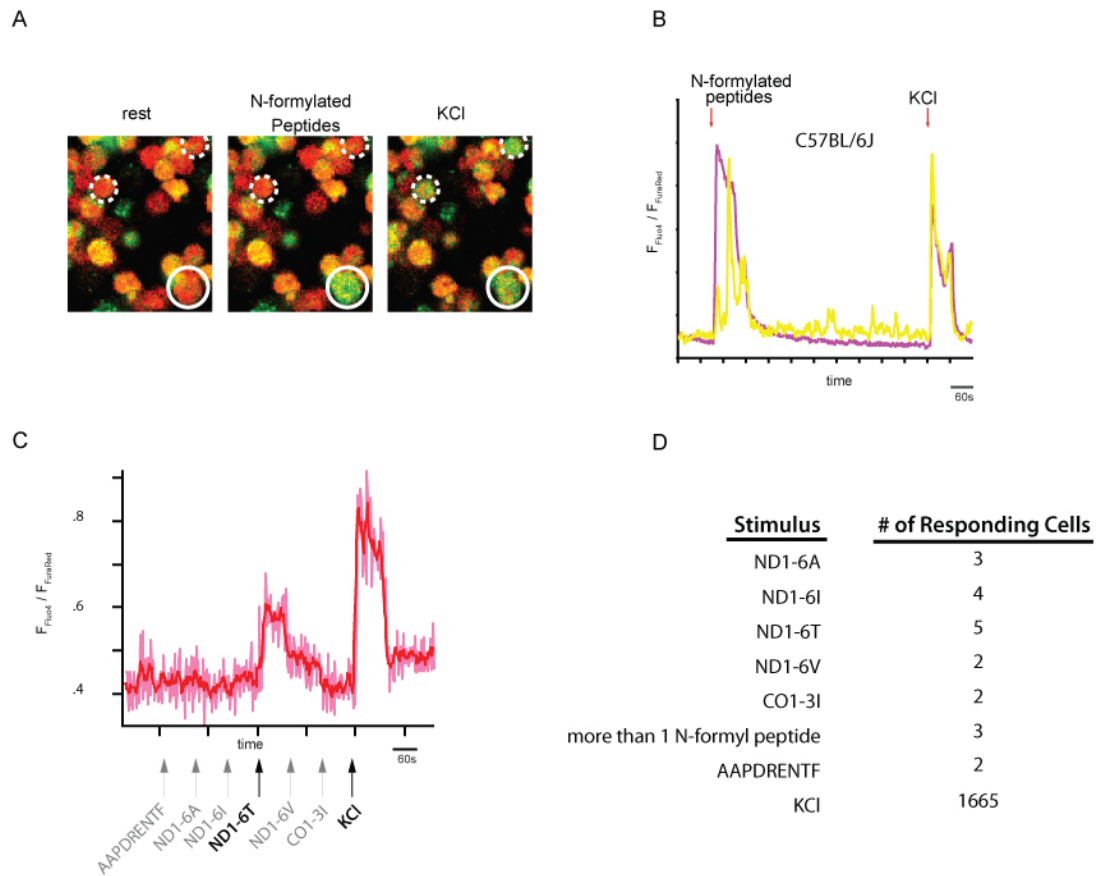


Figure 10: Dissociated VSNs respond to N-formylated Peptides

A) A small number of neurons respond to N-formylated peptides (solid circle) where most (obvious examples in dotted circles) respond to KCl (positive control) but do not respond to N-formylated peptides. B) An example of two cells within one field of view that respond to N-formylated peptides. C) Some neurons selectively respond to N-formylated peptides. D) Only a small number of neurons are responsive to N-formylated peptides.

3.3 GCaMP2 imaging

In order to get a more thorough understanding of VSN responses to N-formylated peptides in the VNO we used mice with a genetically encoded calcium sensor, GCaMP-2, expressed specifically in olfactory marker protein (OMP) expressing cells including OSNs and VSNS (He et al., 2008; Nakai et al., 2001). Within each slice preparation only a small number of neurons responded supporting our data in dissociated neurons that suggests only a small number of neurons are responsive to these peptides (Figure 11). Interestingly clear responses were observed in the basal region of the VNO, suggesting that V2Rs may also detect N-formylated peptides. We found a variety of responses to these peptides (Figure 12). Notably, some neurons appeared more narrowly tuned with respect to the CO1 peptides. Also, with the exception of a single cell (#12), the neurons that respond to ND1 peptides do not appear responsive (or as responsive) to non-formylated versions of the peptides.

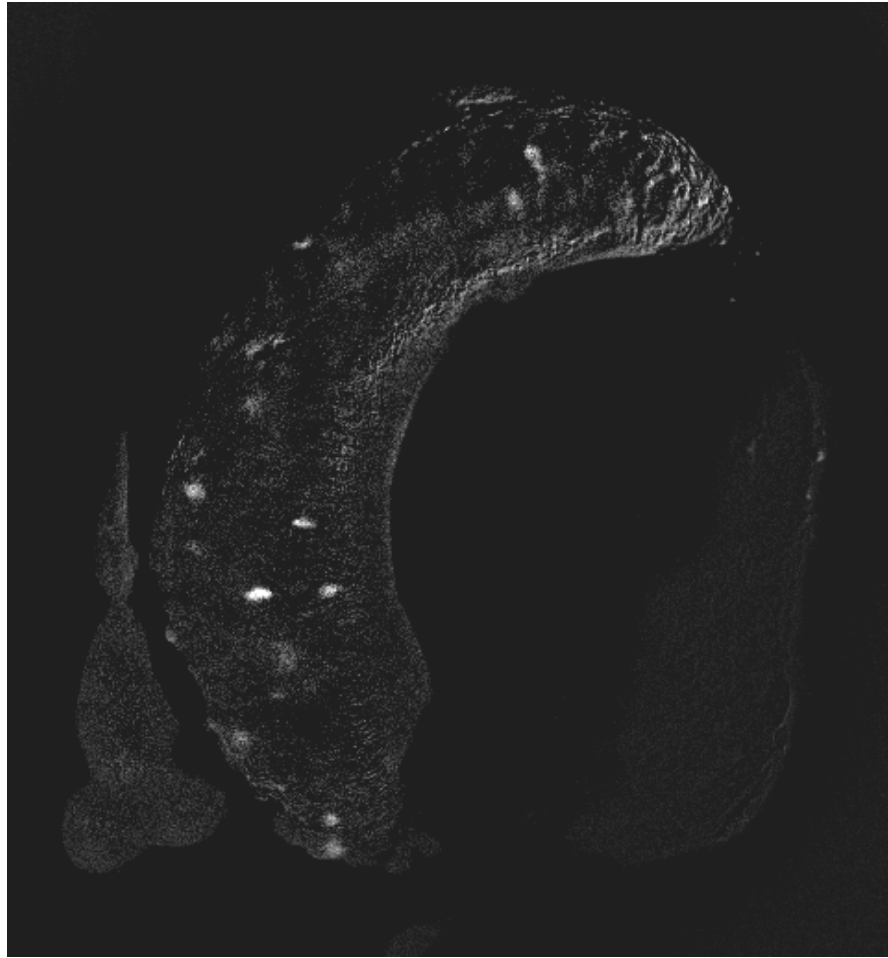


Figure 11: Calcium Imaging in GCaMP2 VNO

Coronal sections of VNO are bathed and imaged as different peptides are applied. Bright areas are responsive neurons.

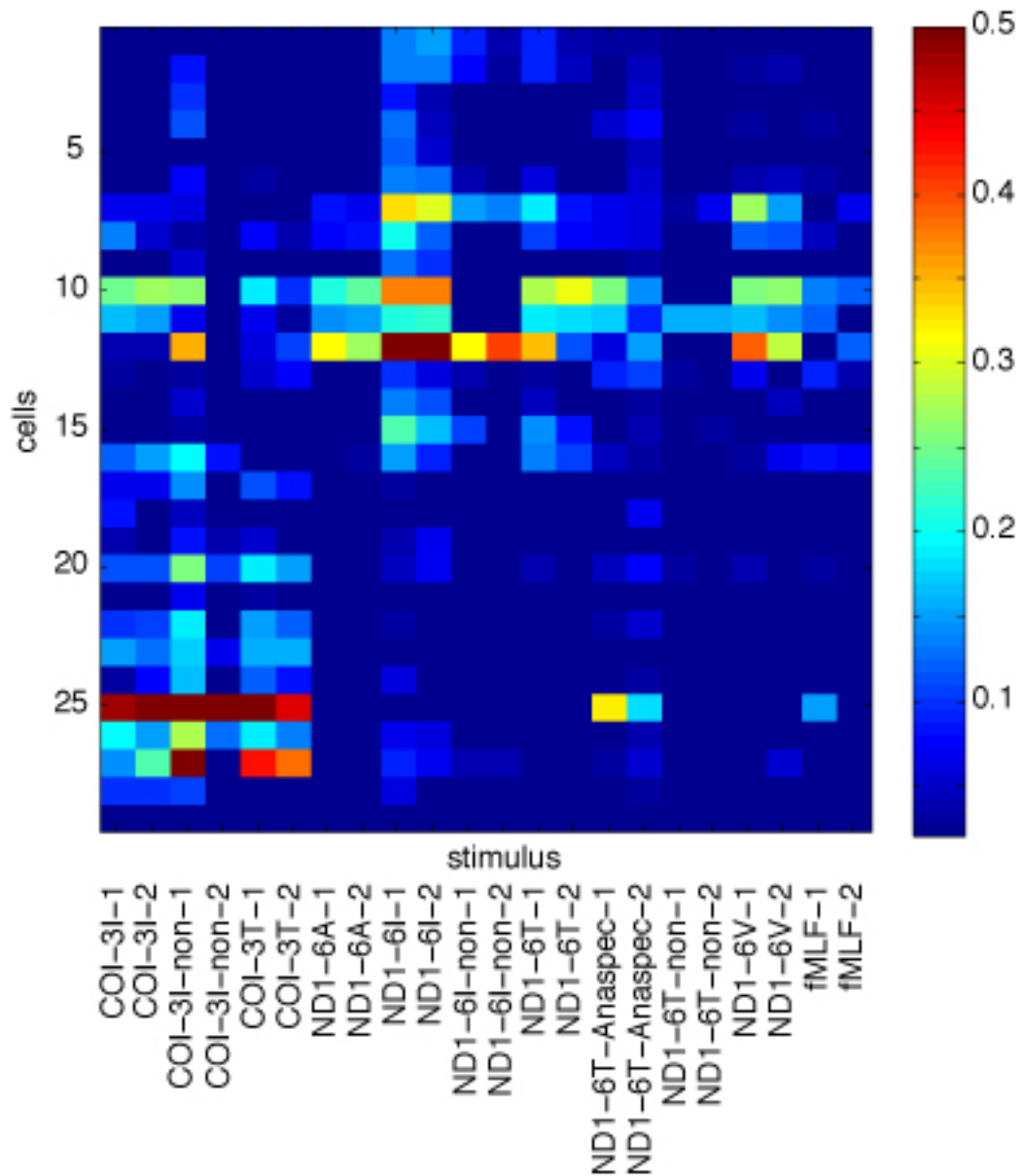


Figure 12: Heat Map of a Representative GCaMP2 Imaging Experiment
 Different peptides (Genscript) are applied in duplicate, represented by -1 and -2. Also "non" refers to non-formylated and Anaspec refers to a ND1-6T peptide from a different vendor.

4. Strain specific responses in a heterologous cell system

Next, we used a heterologous expression system to ask whether FPRs expressed in the VNO are activated by the N-formylated peptides.

4.1 Cell Surface Expression of FPRs in HEK293T Cells

Since it is known that most chemosensory receptors are not efficiently trafficked to the plasma membrane in heterologous cells, we first examined the cell-surface expression of individual FPRs in HEK293T cells. Each family member was tagged on the N-terminus with the first 20 amino acids of human rhodopsin (Rho-tag). Immunostaining of non-permeabilized cells using antibodies against the Rho-tag showed clear cell-surface expression of the FPRs (Figure 13).

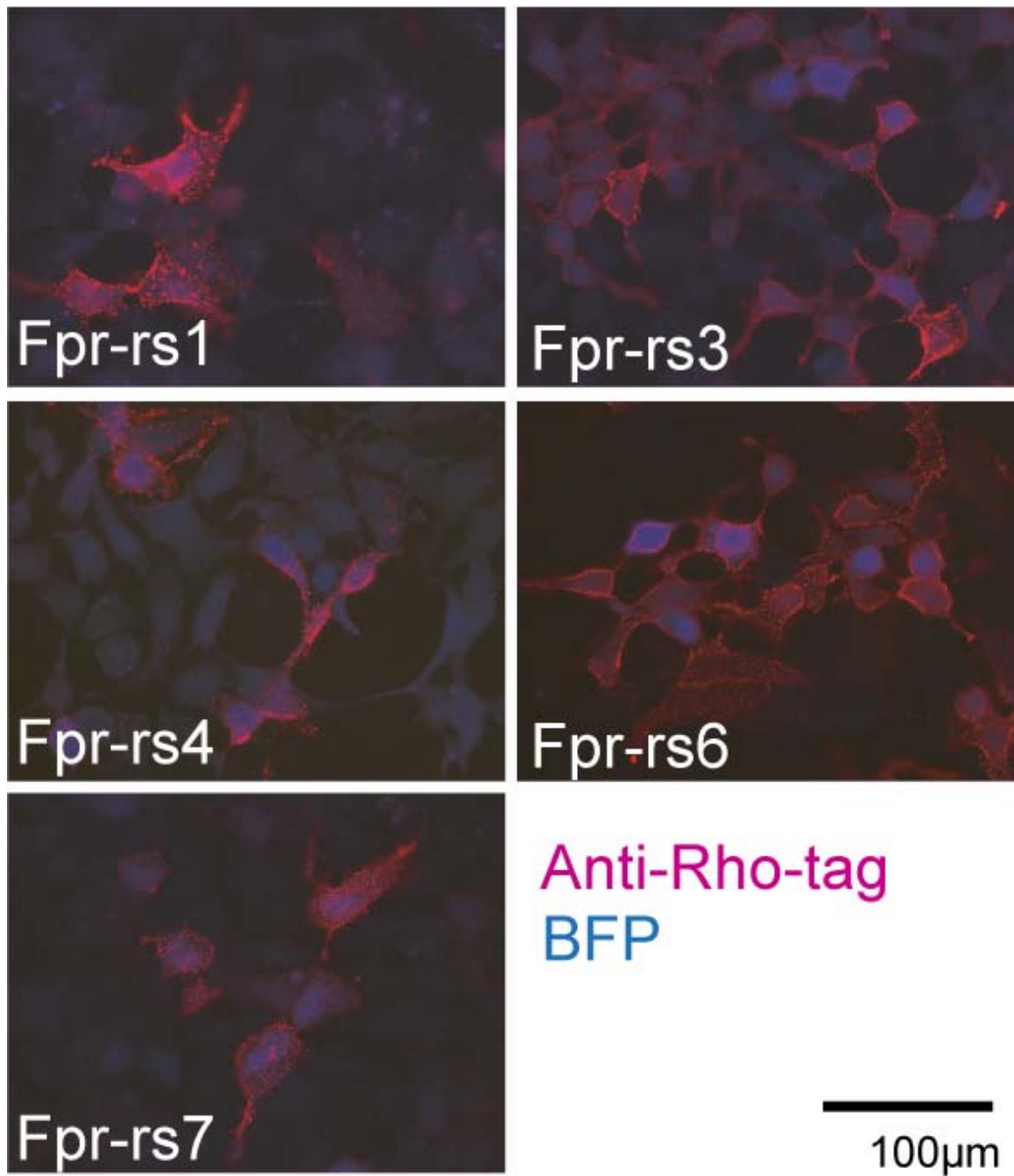


Figure 13: Cell Surface Expression of FPRs

FPRs Are Expressed at the Cell Surface in a Heterologous Cell System HEK293T cells were transfected with BFP and a single Rho-tagged FPR. Antibodies against the Rho-tag were used on non-permeabilized cells.

4.2 Differential Response to MHC peptides

To test the FPR response to N-formylated peptides we performed ratiometric calcium imaging of cells transfected with Rho-tagged FPRs, together with $G_{\alpha 15}$ which couples many, but not all, GPCRs with the phospholipase $C\beta$ (PLC- β) signal transduction pathway that leads to a transient intracellular calcium release (Figure 14) (Kostenis et al., 2005; Offermanns and Simon, 1995; Zhuang and Matsunami, 2007).

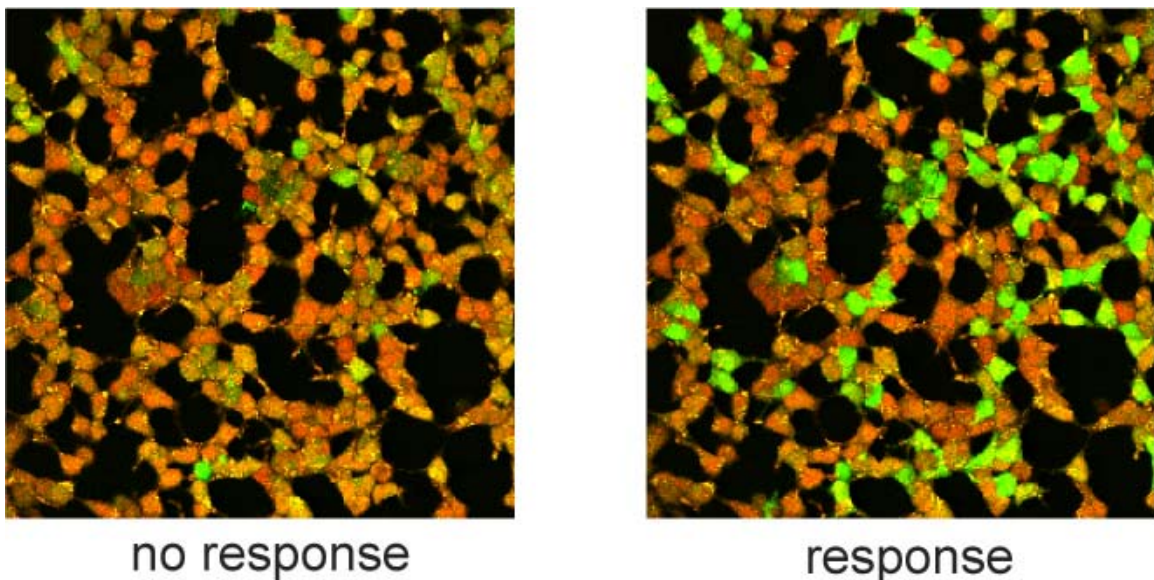


Figure 14: Calcium Imaging

Examples of stimuli responses that elicit either no response or a calcium response

Cells transfected with Fpr-rs1 responded to a subset of those peptides tested (Figure 15 A). Interestingly, of the four variants of ND1 we only observed a Ca^{2+} response in those cells exposed to ND1-6A, or ND1-6T, but not to ND1-6I or ND1-6V at the concentrations we tested (Figure 15 B).

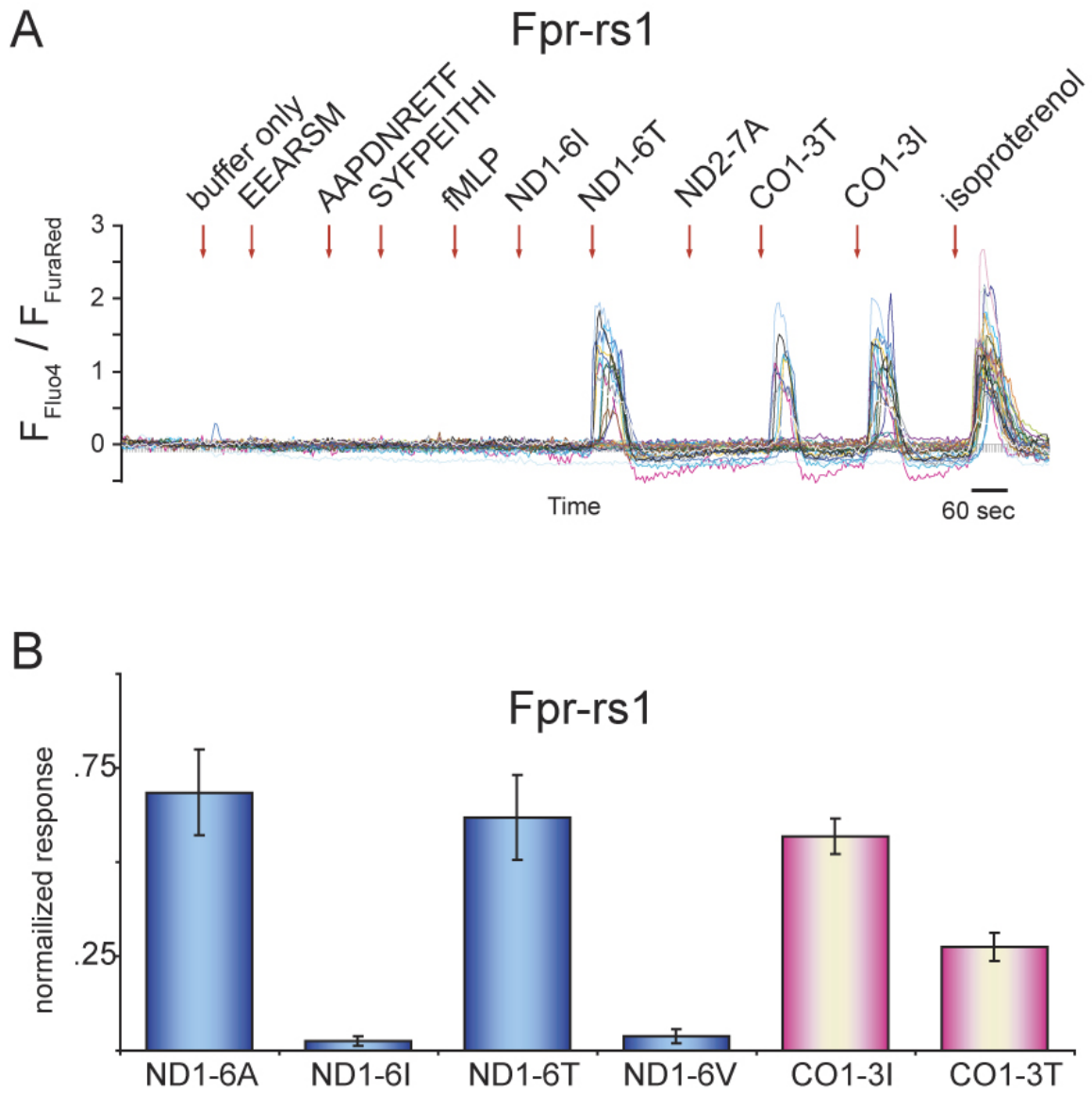


Figure 15: Fpr-rs1 Differential Response to ND1 and CO1

A) A trace profile of 32 isoproterenol (positive control) responding cells in response to a variety of different peptides. Responses only resulted from some ND1 and CO1 peptides. B) Quantification of fpr-rs1 relative responses to ND1 and CO1 peptides.

Though the two allelic variants of CO1 both activate Fpr-rs1, the response to CO1-3T is weaker than CO1-3I, showing 7.6 fold differences in their EC50 values (1.8 μM for CO1-3I and 13.7 μM for CO1-3T, $F(1,13)=14.53$, $P<.0022$, Figure 16).

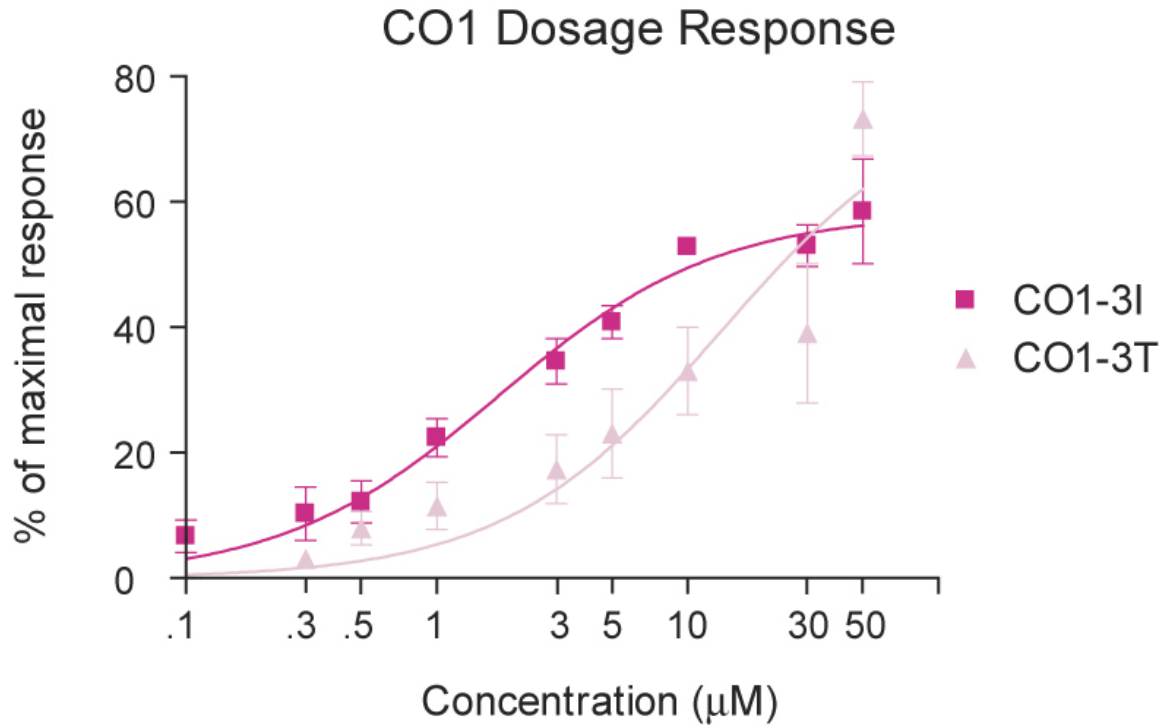


Figure 16: Fpr-rs1 responds differently to the two allelic variants of CO1

We found that the N-formyl-methionine of ND1-6T, CO1-3I and CO1-3T was necessary for receptor activation as non-formylated peptides did not activate the receptor (Figure 17 A). In contrast, when we tested two variants of a mitochondrially encoded N-terminal peptide not known to be presented by H2-M3, peptides derived NADH dehydrogenase subunit 2 (Formyl-MNPITLXII, where X in the 7th position is either A or T), we did not observe an Fpr-rs1 response (Moreno-Loshuertos et al., 2006). In addition, we found that Fpr-rs1 was not activated by the prototypical bacterial peptide, formyl-Methionine Leucine Phenylalanine (fMLF, also known as fMLP), EEARSM, a major urinary protein associated peptide, nor SYFPEITHI or AAPDNRETF, two classical MHC related peptides that are reported to stimulate VSNs (Figure 17 B) (Leinders-Zufall et al., 2004; More, 2006). None of the peptides tested elicited a calcium response in HEK293T cells transfected with either an empty control vector (Rho-pCI, Figure 17 C), or a V1R (V1RF2, data not shown). Using these methods we were unable to identify ligands for other VNO FPRs.

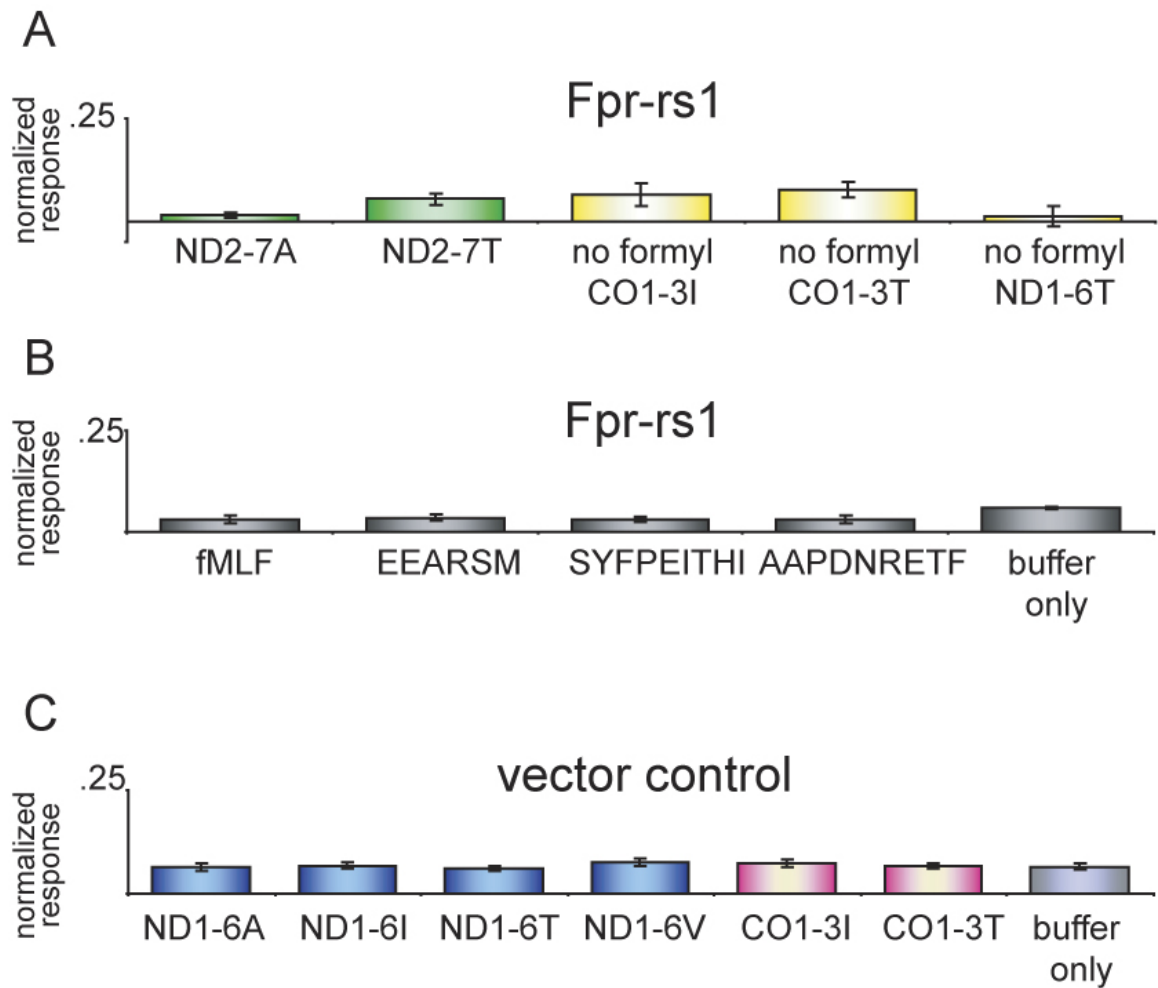


Figure 17: Fpr-rs1 responses are specific

A) Fpr-rs1 does not respond to other polymorphic peptides from the mitochondria that are not known to be displayed by H2-M3, or non-formylated versions of ND1 or CO1 peptides. B) Nor does fpr-rs1 respond to the peptides fMLF, EEARSM, or two other MHC associated peptides that are thought to stimulate the VNO. C) A negative control that does not express Fpr-rs1 is not responsive to any stimuli.

4.3 Differential Responses between Strains

Next we wanted to know if strain specific polymorphisms in the *Fpr-rs1* receptor could alter the response profile to N-formylated peptides. Sequencing *Fpr-rs1* from BALB/cJ and NZB/B1NJ mice demonstrated that there were three N-terminal polymorphisms shared between the two strains. Additionally, BALB/cJ mice have a four amino acid deletion just prior to the fourth transmembrane spanning region, a polymorphism described previously in a 129 strain (Wang and Ye, 2002). Ten other amino acid changes were found in the SNP database at NCBI. Responses to N-formylated peptides were the same in C57Bl/6J mice and NZB/B1NJ mice. Interestingly the four amino acid deletion found in the BALB/cJ mice abrogated response to a 30uM mix of ND1-6T, CO1-3I, and CO1-3T peptides (Figure 18).

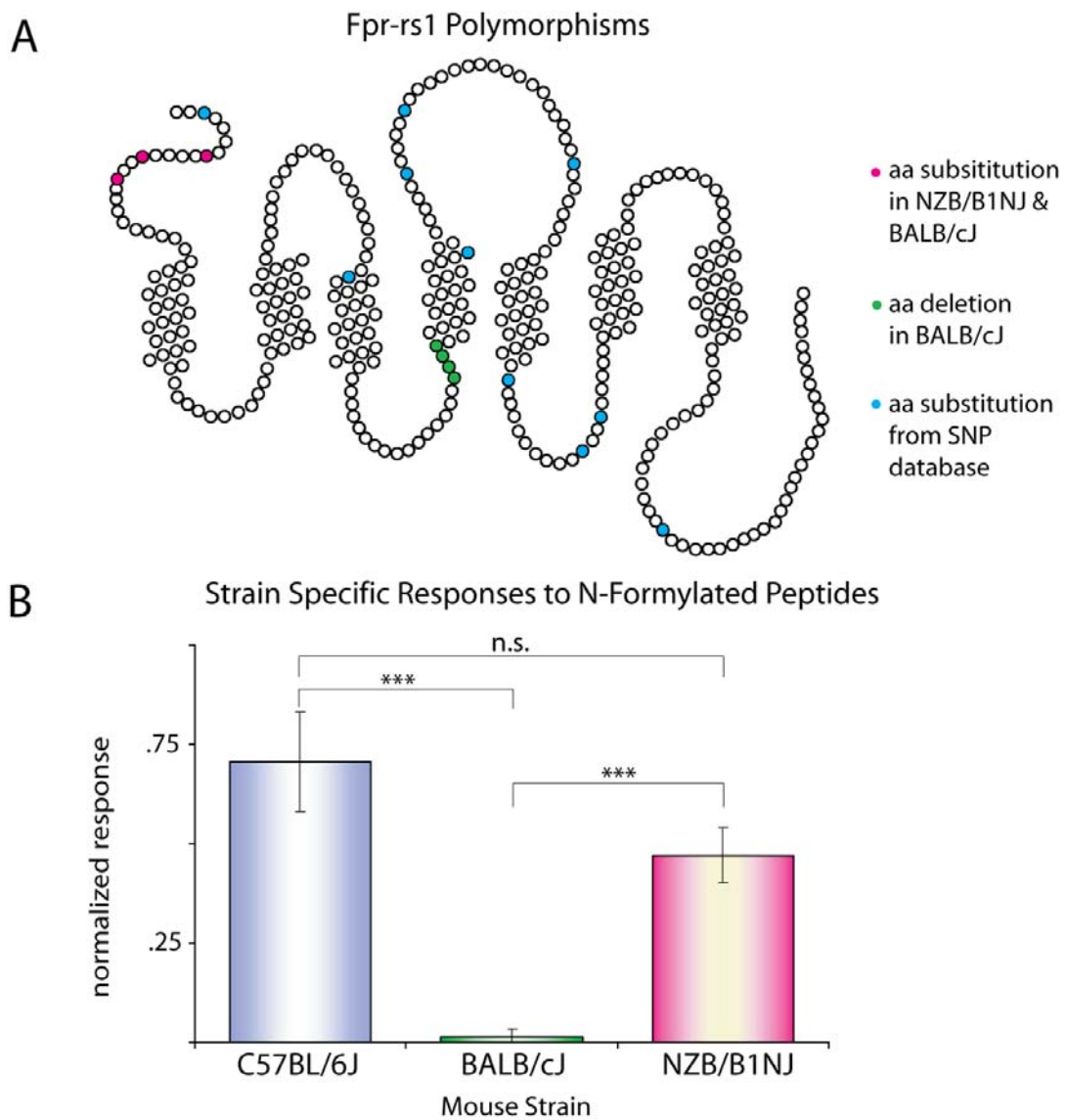


Figure 18: Fpr-rs1 from BALB/cJ is not responsive to N-formylated Peptides
 A) The location of variations from the C57Bl/6J amino acid sequence. B) The relative responses to N-formylated peptides by different strains. Fpr-rs1 from BALB/cJ does not respond to these peptides.

5. Physiological Consequences of H2-M3 peptides

Given that N-formylated peptides differentially activate Fpr-rs1 and stimulate the VNO, they could signal individual information via the vomeronasal system. This possibility can be tested in the context of the pregnancy block effect, also known as the Bruce effect. In this behavioral paradigm a high incidence of pregnancy failure occurs when recently mated female mice are exposed to chemosignals from an unfamiliar male (Bruce, 1959). The terminating signal of the unfamiliar male is mediated by the accessory olfactory system, resulting in a suppression of the prolactin and progesterone levels required for embryonic implantation (Bellringer et al., 1980; Brennan, 2004; Li et al., 1994). The mating male has the capacity to terminate pregnancies by a male of a different strain, but does not block pregnancies that he himself has induced. This is because the female recognizes the individual identity of her mate's chemosignals during a sensitive period at the time of mating and gates their effects so as to maintain the pregnancy (Keverne and de la Riva, 1982).

5.1 Pregnancy Block

BALB/c female mice were mated with BALB/c males and then exposed to urine taken from either BALB/c or CBA males. Application of the unfamiliar CBA urine resulted in a high level of pregnancy block, whereas the familiar BALB/c urine did not (Figure 19, groups 1 and 2). The effect of unfamiliar CBA urine could be mimicked by adding the N-formylated peptides, ND1-6I, an ND1 variant derived from CBA mice,

or ND1-6A from the NZB strain, to familiar BALB/c urine samples at 50µM (Figure 19, groups 3 and 4). Thus familiar male urine could be converted to unfamiliar urine by the addition of a single N-formylated peptide. In contrast, when ND1-6T derived from BALB/c was added to BALB/c urine samples at an equimolar concentration, it did not cause a high level of pregnancy block (Figure 19, group 5).

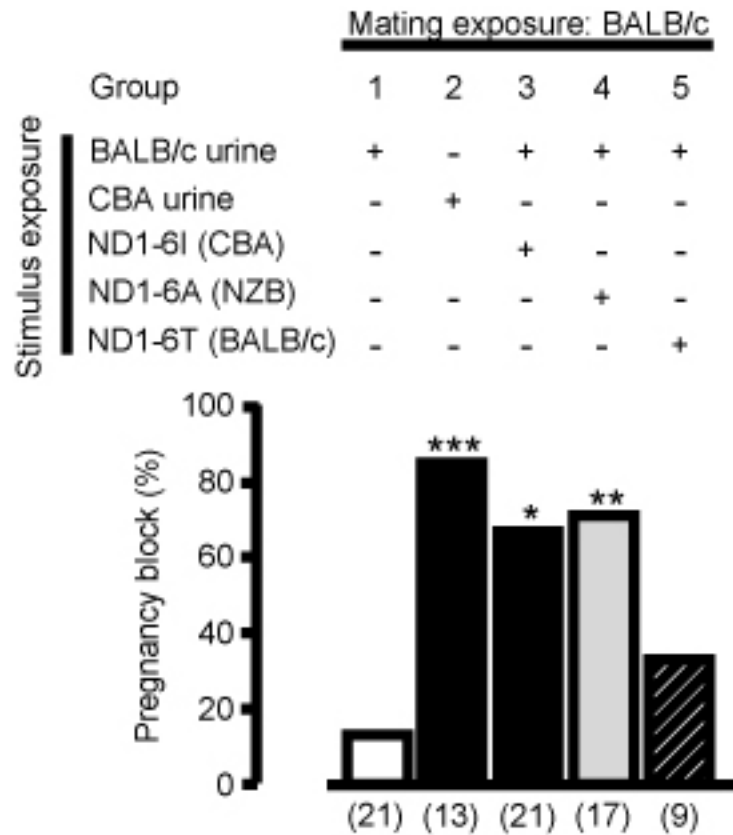


Figure 19: Pregnancy Block after mating to BALB/c males

BALB/c females were mated with BALB/c males and then exposed to different stimuli including the negative control BALB/c urine, the positive control CBA urine, and different strain specific peptides added to the negative control.

Similar effects with ND1-6A and ND1-6T were observed when castrated BALB/c male urine was used (Figure 20).

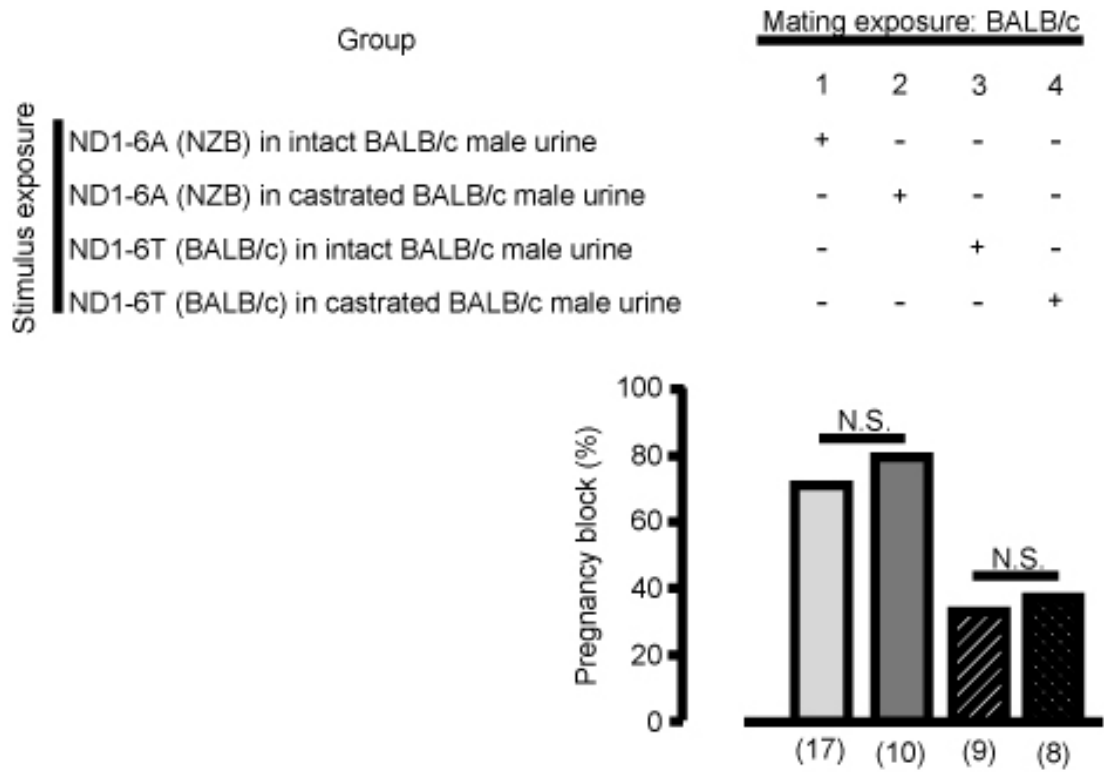


Figure 20: Castration does not affect pregnancy block

When BALB/c females were mated with CBA males, pregnancy block occurred after the application of BALB/c urine, but not of CBA urine (Figure 21, groups 1 and 2). In this case addition of ND1-6I derived from CBA to CBA urine did not alter the native response to CBA urine, resulting in a low level of pregnancy block in CBA-mated BALB/c females (Figure 21, group 3), However, though ND1-6A derived from NZB blocked pregnancy, ND1-6T derived from BALB/c was ineffective at blocking pregnancy (Figure 21, groups 4 and 5). Taken together these results suggest that N-

formylated peptides can function as individuality chemosignals, and that response to these peptides varies between strains.

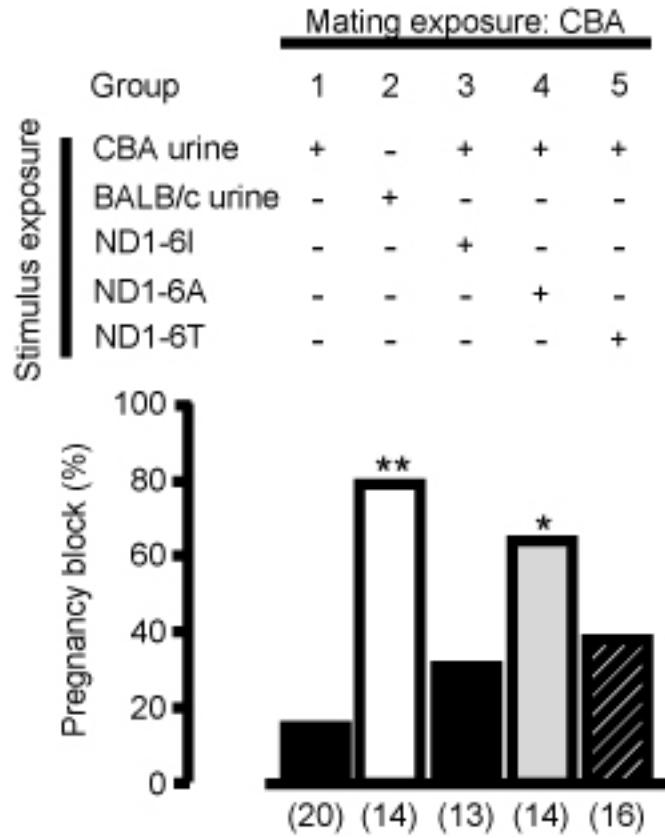


Figure 21: Pregnancy Block after mating to CBA males

BALB/c females were mated with CBA males and then exposed to different stimuli including the negative control CBA urine, the positive control BALB/c urine, and different strain specific peptides added to the negative control.

6. Discussion

6.1 FPRs as chemosensory receptors

The descriptive signals of an individual are detected by chemosensory receptors in two distinct chemosensory pathways, the main olfactory and accessory olfactory systems. Olfactory sensory neurons in the main olfactory epithelium utilize two receptor types, ORs and TAARs, to detect a vast array of chemicals including individuality cues (Buck and Axel, 1991; Liberles and Buck, 2006; Lin et al., 2005). A small subset of olfactory neurons utilizes guanylyl cyclase type D (GC-D), a single transmembrane receptor, to detect CO₂ and peptides (Hu et al., 2007; Leinders-Zufall et al., 2007; Sun et al., 2009). The accessory olfactory pathway originates from the VSNs in the VNO. The VSNs express two known putative pheromone receptor families, the V1Rs and V2Rs, in two molecularly and spatially distinct regions (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Here we describe a putative third class of chemosensory receptor, the FPRs, expressed in the VNO. It is possible that the FPRs are expressed in other untested chemosensory tissues. Expression of FPRs in the Grueneberg ganglion and septal organ was not investigated in this study (Ma, 2007; Olson et al., 2005).

Though the two olfactory systems by which mice receive a plethora of chemical cues are both molecularly and anatomically distinct, the receptor types do share some common characteristics (with the exception of GC-D). First, each receptor family is composed of seven-transmembrane GPCRs expressed in distinct populations of neurons within the MOE or the VNO. Second, the genes encoding each receptor type are organized in clusters. Third, the receptor families are undergoing

rapid evolutionary change, with extensive gains and losses of genes. Notably, the chemosensory receptor family repertoire is significantly larger in the rodent species such as mouse and rat than in the primates such as human, chimpanzee and rhesus macaque, likely reflecting the relative importance of chemical cues in each lineage (Young et al., 2002). For example, based on recent estimations, the mouse genome encodes 1035 ORs, 15 TAARS, 191 V1Rs and 61 V2Rs, whereas humans have only 387 ORs, 5 TAARS, 2 V1Rs and no V2Rs (Hashiguchi and Nishida, 2007; Niimura and Nei, 2007; Yang et al., 2005; Zhang et al., 2007). The FPRs we have identified in the VNO share characteristics with known chemosensory GPCRs. Future work is necessary to determine whether the human FPRs have any chemosensory function. Whether other chemosensory GPCRS remain to be discovered is unclear. We would expect that if other receptors exist they, like the FPRs, account for a small percentage of OSNs or VSNs.

6.2 Mitochondrially-encoded formyl peptides as VSN ligands

VSNs are activated by a wide variety of chemicals including pheromones and individual-specific signals (Chamero et al., 2007; He et al., 2008; Kimoto et al., 2005; Kimoto et al., 2007; Leinders-Zufall et al., 2004; Leinders-Zufall et al., 2000; Luo et al., 2003). Some apical VSNs are stimulated by small molecules that can alter the oestrus cycle, delay puberty, or induce aggression (Jemiolo et al., 1989; Jemiolo et al., 1986; Leinders-Zufall et al., 2000; Novotny et al., 1986; Novotny et al., 1999a; Novotny et al., 1999b). Some neurons in the basal layer detect peptide cues, including MHC presented peptides, exocrine gland secreting peptides (ESPs), and major urinary proteins (MUP) complexes (Chamero et al., 2007; Kimoto et al., 2005;

Kimoto et al., 2007; Leinders-Zufall et al., 2004). We show here that two polymorphic alloantigenic peptides ND1 and CO1, mitochondrially-encoded peptides presented by the nonclassical MHC class 1b molecule H2-M3, stimulate VSNs. The percentages of cells responding to N-formylated peptides in calcium imaging experiments (.96%) as well as the response profiles of individual neurons are consistent with the idea that a small number of receptors are differentially activated by variants of N-formylated peptides. Furthermore, we demonstrate that Fpr-rs1 is one such receptor. However, both the calcium imaging and behavioral data suggest that Fpr-rs1 is only one of multiple receptors – that could include other FPRs, V1Rs, V2Rs, or other unidentified receptors - activated by variants of N-formyl peptides. These formyl peptides are unique among the VSN ligands, because they are polymorphic and maternally inherited. Together these two traits provide the means for animals to discern an individual's maternal lineage.

Though H2-M3 expression is ubiquitous it is not clear what the biological source of these peptides is. The hydrophobic nature of these peptides, in addition to the complex nature of biological samples, present challenges to the identification of these peptides in a biological context. Furthermore, given the social nature of chemosignaling it may be that release of N-formylated peptides is itself governed by an olfactory cue. Given the behavioral impact of these peptides when presented in urine one might hypothesize that these peptides are deposited into the urinary tract. However, urine may not be the only source of cues that cause pregnancy block, as saliva from strange males is also shown to block pregnancy (Thompson et al., *AchemS XXXI*). The original experiments leading towards the identification of H2-M3 and the formyl peptide ligands involved skin grafts, indicating that the peptides are

presented by skin cells. Thus, it is also plausible that these peptides are deposited on the surface of an animal's skin.

6.3 Roles of mitochondrially-encoded formyl peptides in chemical communications

We show that formyl peptides are capable of eliciting physiological responses in selective pregnancy block. It is plausible that N-formylated peptides activate chemosensory receptors other than FPRs. Indeed, our results support a model where receptors other than Fpr-rs1 are activated by ND-1 peptides as ND1-6I is capable of inducing a pregnancy block in a BALB/c female but does not elicit a calcium response in Fpr-rs1 transfected cells. Similarly, Fpr-rs1, as well as other VNO FPRs, could be activated by non-formylated peptides, untested peptides, or non-peptide ligands. Lymphocyte FPRs are known to be activated by acetylated N-terminal peptides, other peptides, and lipid metabolites (Le et al., 2002; Pamer et al., 1992; Shawar et al., 1991; Vyas et al., 1992). It should be noted that the interpretation of a lack of activity in an *in vitro* cell system cannot be extended to *in vivo* systems. Others recently showed that the VNO FPRs were activated by antimicrobial peptide CRAMP, uPAR, lipoxin A4, a prototypical bacterial peptide fMLF and that these ligands activated a subset of VNO neurons (Riviere et al., 2009b).

Our results offer a class of maternal identity cues that can be discriminated by receptors in the VNO. We demonstrate that the inclusion of a single N-formylated peptide to the urinary cues of a familiar male is sufficient to convey the chemosensory information of an alien male. Our experimental conditions use male or

castrated male urine as a vehicle to deliver N-formylated peptides. We do not intend to suggest that these mitochondrial peptides are the sole inducers of a pregnancy block. Rather, N-formyl peptides, as well as other individuality cues such as classic MHC-presented peptides, can carry strain specific information in the context of the selective pregnancy block. Others have been successful inducing the termination of pregnancy by the addition of a mixture of MHC class 1a peptides (Leinders-Zufall et al., 2004). It remains unclear whether these cues affect some or all of the same set of receptors activated by N-formylated peptides or utilize alternative pathways. Future experiments including the behavioral or physiological consequence of specifically activating or inactivating one or more members of the FPR receptors in the VNO and the use of non formylated versions of the mitochondrially-encoded peptides in the behavioral assays will help address this issue. Additionally, maternally inherited peptides may serve other functions. For example, a study of F1 mice from reciprocal crosses of unrelated strains, mice were found to avoid urine from individuals that were the same strain as the mother, potentially encouraging outbreeding to increase genetic diversity, and fitness (Isles et al., 2001).

6.2 Connection between chemosensory and immune systems

The role MHC family members may have in chemosensory signaling is increasingly complex. It is known that MHC peptides stimulate neurons in both the VNO and the MOE (Leinders-Zufall et al., 2004; Spehr et al., 2006). Here we demonstrate that another class of alloantigenic or “self-recognition” peptides from the immune system can be used to provide identity information as an olfactory signal to other individuals. In this regard, it is intriguing that the FPRs might respond to

both strain-specific N-formylated peptides and peptides related to infection (Riviere et al., 2009a).

The non-classical H2-M1 and M10 family members are expressed in the VNO and proposed as possible chaperones or co-receptors functioning with the V2Rs (Loconto et al., 2003). The H2-M3 gene, located on the same gene cluster as M1 and M10, is not specifically expressed by the VSNs but widely expressed in other tissues (Loconto et al., 2003). The H2-M3 gene product has a critical role in presenting N-formylated peptides that can stimulate the VSNs. One could speculate that the M1 and M10 families, the most closely related MHC molecules to H2-M3, could also interact with N-formylated peptides, though there is no evidence supporting this hypothesis (Olson et al., 2005).

Likewise, it is interesting to consider whether other chemotactic receptors may have been co-opted by chemosensory systems for the detection of olfactory cues. In evolutionary terms the reuse of existing systems is certainly more parsimonious than assembly of new ones. It will be important to assess how MHC genes have evolved in the context of both immune and chemosensory functions.

7. Experimental Procedures

7.1 *In situ hybridization*

RNA probes were labeled by either Dig or fluorescein (Roche) and hybridized at 58°C or 50°C (Ishimaru et al., 2006). Dig labeled probes were detected by HRP conjugated anti Dig (Roche) followed by TSA-plus FITC (Perkin-Elmer). For two-color *in situ* hybridization fluorescein-labeled probes were detected by HRP conjugated anti fluorescein (Roche) followed by TSA-plus Cy3 (Perkin-Elmer). HRP was inactivated by incubating with PBS containing 3% H₂O₂ for 30 min, followed by detection of Dig labeled probes.

7.2 *RT-PCR*

Sixteen different mouse tissues were dissected and isolated separately. Total RNA was extracted from these tissues using TRIzol Reagent (Invitrogen), and purified with RNeasy Mini kit (Qiagen), then reverse transcribed into cDNA using oligo dT primer and SuperScript II Reverse Transcriptase (Invitrogen). ~500 bp coding regions encompassing multiple exons of FPRs and GAPDH were amplified from each cDNA for 30 cycles with specific primers using HotStarTaq DNA Polymerase (Qiagen). The volume of PCR products loaded on the agarose gel was approximately normalized to the PCR products of GAPDH.

7.3 *EVG recordings*

Local field potentials were recorded from the sensory epithelium of extracted VNOs with stimulants focally applied in 500 ms pulses as described by others. Buffer

solution containing 120mM NaCl, 25mM NaHCO₃, 5mM KCl, 5mM BES, 1 mM MgSO₄, 1mM CaCl₂, 10mM Dextrose, and superfused with 95% O₂, 5% CO₂ was used for tissue extraction, a continuous bathing, and dilution of stimulants (Kelliher et al., 2006). Output signals were digitized, highpass filtered at .1Hz, lowpass filtered and 50 Hz, and analyzed with Igor Pro. Responses are representative of 9-17 traces from 3-4 mice.

7.4 Cell culture and Calcium imaging

Cell culture was performed as previously described (Saito et al., 2004) Expression vectors for FPRs were constructed by subcloning coding regions from C57Bl/6J into pCI (Promega). For calcium imaging, for dissociated VSNs, the VNO was extracted, and dissociated with Papain solution containing 2.2U/ml papain, .5mM EDTA, 5mM cysteine, in PBS. For HEK 293T, the cells were seeded on poly-d-lysine coated glass coverslip bottom plates (MatTek), plasmid DNA was transfected using lipofectamine 2000 (Invitrogen), and incubated for 36-42 hours before dye loading. VSNs were loaded with 4 μ M of Fluo-4 (Invitrogen) and 7 μ M of Fura Red (Invitrogen) for 10-15 minutes at room temperature. HEK293T cells were loaded for 45min. Transfection efficiency was consistent (\sim 50%), as judged by blue fluorescent protein expression. We used Leica confocal microscope (excitation 488nm, emission 500-560nm for Fluo-4, 605-700nm for Fura-red) and the live imaging mode of Leica confocal software for data acquisition. Data were collected at 3s interval. Cells were exposed to constant flow of bath solution (Hank's buffer containing 10mM HEPES, 5mM glucose, Invitrogen). Synthetic peptides were prepared by Sigma Genosys and Genscript. Peptides were stored at 10mM in DMSO and further diluted in bath solution. Peptide solutions were applied to cells for \sim 15s by changing the bath

solution with a peristaltic pump (Rainin, Rabbit) (Sato et al., 1994). For data analysis of dissociated VSNs, we counted the number of the cells responding to a given stimulus and KCl. Each field contained 55-256 KCl-responding cells and 1-2 cells responding to the formylated peptides. For heterologous cells, we randomly chose 25-32 cells that were responsive to isoproterenol from the entire field of ~200 cells. The isoproterenol activates the endogenous β 2 adrenergic receptors, and is independent of FPR overexpression, but dependent on $G_{\alpha 15}$ overexpression. This was used to normalize results between experiments. The maximum response to each stimulus was averaged over all selected cells. Each response was normalized to the isoproterenol response. Results were compiled from three to seven replicates. For dosage response curves peptides were applied in ~200sec intervals of increasing concentration from 0 μ M to 50 μ M. The number of responding cells was counted. Since partial desensitization was observed after repetitive stimulations, we designated a responding cell as any cell that responded to the current stimulus, or responded to a lower concentration. We then normalized the number of responding cells to each stimulus to the total number of respondent cells. Data analysis was done with Image J, Microsoft Excel, and GraphPad Prism.

7.5 Pregnancy block

Animals were adult males and virgin females of the BALB/c strain males (Japan SLC, Hamamatsu, Japan). The animals were housed singly at $21 \pm 1^\circ\text{C}$ with a reversed 12: 12 light cycle (lights on at 2100 hours, lights off at 0900 hours). The estrous cycles of the females were monitored daily by taking vaginal smears. Mating was carried out naturally by placing a single estrous female into the home cage of a

male at 1400 hours and checked for vaginal plugs at 2100 hours when the mating male was removed. Mated females were left in the cages of the mating males until 0800 hours on the day following mating before removal to a clean cage. Exposure to urine or supplemented urine was achieved by depositing 30 μ l of liquid on the oronasal groove while holding the female by the nape of the neck. The females were exposed at 0800, 1400, 2000 and 2400 hours on each of the two days following mating. Seven days after mating the females were killed and their uteri examined for implantation sites. Whole urine was collected from adult males, pooled by strain, and stored at -80°C. N-formylated peptides were stored at 10 mM in DMSO and further diluted in PBS. Urine samples were mixed 1:1 (vol/vol) with peptide solutions (final concentrations: 50 μ M peptides and 0.5% DMSO).

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