# Murine Pheromone Proteins Constitute a Context-Dependent Combinatorial Code Governing Multiple Social Behaviors

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## SUMMARY

During social interactions, an individual's behavior is largely governed by the subset of signals emitted by others. Discrimination of "self" from "other" regulates the territorial urine countermarking behavior of mice. To identify the cues for this social discrimination and understand how they are interpreted, we designed an olfactory-dependent countermarking assay. We find major urinary proteins (MUPs) sufficient to elicit countermarking, and unlike other vomeronasal ligands that are detected by specifically tuned sensory neurons, MUPs are detected by a combinatorial strategy. A chemosensory signature of "self" that modulates behavior is developed via experience through exposure to a repertoire of MUPs. In contrast, aggression can be elicited by MUPs in an experience-independent but context-dependent manner. These findings reveal that individually emitted chemical cues can be interpreted based on their combinatorial permutation and relative ratios, and they can transmit both fixed and learned information to promote multiple behaviors.

## INTRODUCTION

During social behavior, each participant emits a variety of sensory cues. The receiver likely uses multiple neural strategies in order to identify those cues that are sent by others within the milieu of all detected cues. How self-emitted cues are detected and filtered to allow receivers to respond specifically to nonself-cues is largely unknown. In addition to direct interaction with conspecifics, male mice also communicate by proxy: they deposit urine odor cues in the environment to advertise their presence to females and rival males (Desjardins et al., 1973; Rich and Hurst, 1999). If another male's mark is encountered by a dominant male, he will reply with a "countermark" to indicate command of the territory (Rich and Hurst, 1999). This behavior is

metabolically costly; therefore, contact with a self-deposited mark does not initiate marking behavior (Nevison et al., 2000). Identification of the behavior-promoting ligands, the olfactory strategy that enables the discrimination between self and other, and the responding sensory neurons will provide a tractable system to begin to address the neural mechanisms that distinguish self from other.

Instead of being tuned to a specific ligand, main olfactory neurons detect molecular features of odorants (Malnic et al., 1999). Therefore, depending on the diversity of its molecular features, each ligand activates multiple sensory neurons, and each neuron detects multiple ligands, termed "combinatorial coding." This strategy enables a limited number of receptors to capture a large amount of information. The main olfactory system functions to recognize the identity of the odor blend through the composition of its repertoire and does not easily discriminate individual odorants. In contrast, stimulation of the vomeronasal organ (VNO) has been shown to mediate identical behavioral responses whether the ligand is purified or in the context of a native odor blend (Kimoto et al., 2005). This difference may enable the VNO to initiate fixed responses to specialized ligands. The bioactivity of very few VNO ligands has been solved. Purifying additional ligands and solving their function are necessary to study how this sensory system evaluates the environment.

Mouse urine is composed of a large number of volatile odors as well as peptides and proteins that function as chemosignals to promote social behavior. A subset of proteins, major urinary proteins (MUPs), is produced in a testosterone- and growth hormone-dependent manner primarily by adult males (Finlayson et al., 1965; Hastie et al., 1979; Knopf et al., 1983; Szoka and Paigen, 1978). MUPs have been shown to be detected by vomeronasal sensory neurons (VSNs) (Chamero et al., 2007, 2011; Papes et al., 2010). In contrast to main olfactory neurons, VSNs have been found to be tuned to specific cognate ligands (Haga et al., 2010; Leinders-Zufall et al., 2000; Nodari et al., 2008). This requires evolution of a unique receptor for each ligand. The mouse reference genome encodes 21 MUPs, all species specific, 15 of which are extremely similar, with some proteins varying by only a single amino acid (Logan et al., 2008;

Mudge et al., 2008). These observations are consistent with a rapidly evolving gene family. It is not known whether such ligands can be uniquely distinguished by coevolving sensory neurons or if they are detected by a limited number of VSNs that would render the individual gene products functionally redundant. As evidence against redundancy, an individual does not express all of the 21 MUPs; rather, individual males stably express discrete subsets of 4–12 of the MUPs throughout their lifetime (Robertson et al., 1997). Although wild-caught brothers each emit a unique MUP profile, all inbred males of the same strain emit identical MUPs, and males of other strains may express a different MUP subset (Cheetham et al., 2009). Why individuals express varying repertoires of these specialized ligands is not known.

Recombinant MUPs (rMUPs) have been shown to promote male-male territorial aggression (Chamero et al., 2007), female attraction, and conditioned place preference (Roberts et al., 2010, 2012). MUPs have additionally been proposed to play a role in signaling individual identity for countermarking behavior based on three observations. (1) MUPs are lipocalins, which fold into degradation-resistant β-barrel structures that effectively persist in the environment (Flower, 1996; Hurst et al., 1998). (2) Male mice emit an extraordinarily high MUP concentration (20 mg/ml) in their urine (Szoka and Paigen, 1978). Protein excretion in urine is unusual in mammals due to high metabolic cost, suggesting that their function is likely to be a species-specific evolved trait. (3) The unique MUP repertoire of each individual is stable throughout his lifetime and has been proposed to be a potential protein "bar code" of individuality (Hurst et al., 2001). Indeed, male mice increase their marking when they encounter MUP-containing urine fractions (Humphries et al., 1999). Males can discriminate between native urine and the same urine spiked with rMUPs (Hurst et al., 2001). However, the role of MUPs in countermarking may be indirect because the β-barrel structure of MUPs binds volatile urine molecules (Bacchini et al., 1992; Novotny et al., 1999), retaining them in the environment, extending their potency as volatile odor cues (Hurst et al., 1998), and transporting them into the mucous-filled VNO lumen that is otherwise not readily accessible to volatiles (Meredith and O'Connell, 1979). These MUP-associated ligands are sufficient to activate VNO neurons and promote social behavior (Leinders-Zufall et al., 2000; Novotny, 2003). Whether mice detect MUP type to promote countermarking through differences in volatile ligands, through simultaneous detection of MUPs and their ligands, or through the MUPs themselves has not been determined (Hurst and Beynon, 2004; Hurst et al., 2001). Furthermore, how MUPs can be necessary to regulate a variety of disparate social behaviors is not known.

The large MUP repertoire provides an experimental tool kit to investigate the sensory logic underlying social behavior. Here, we fractionate urine to identify the underlying bioactive cues and confirm that the MUP fraction elicits countermarking. We further assay recombinant proteins to determine that the MUPs alone, not the bound odor molecules, are each relevant to promote countermarking behavior. We use both calcium ($Ca^{2+}$) imaging and electrophysiology and find that VSNs employ a combinatorial-coding strategy to sense and interpret the identity and concentration of MUPs in the environment. Surprisingly, we find that MUP bioactivity to instruct countermarking behavior depends not on individual MUP ligands but on the blend of the entire detected MUP repertoire as a whole. Through behavioral manipulations, we demonstrate that the ability of the encountered MUPs to signal "self" or "other" varies with previous MUP sensory experience. In contrast, we find that two particular MUPs are predetermined to innately elicit male-male aggression, a stereotyped output that is not modulated by concentration, experience, or the entire detected MUP repertoire. Through behavioral analysis, we show that the decision to respond to detected MUPs with either aggression or countermarking depends upon the extended sensory context. Overall, we find that males use MUP ligands to regulate two different behaviors, each with a different sensory-coding strategy. Aggression is highly tuned and is promoted by dedicated ligands. In contrast, countermarking utilizes combinatorial-sensory coding, and the propensity of each ligand to promote behavior varies based on the experience of the receiving animal.

## RESULTS

### MUPs Are Sufficient to Promote Countermarking Behavior

To isolate the urinary cues that promote and regulate countermarking, we devised an olfactory-mediated behavioral assay. BALB/cByJ male mice were placed in an empty cage lined with Whatman paper spotted with 50 μl of an olfactory stimulus. After 5 min, the animal was removed, and urine marks revealed by ninhydrin treatment were quantified (Figures 1A and 1B). The cues that signal self are likely to contain a genetic component because it has previously been shown that the marking response to urine from any male of the same inbred strain is identical to that elicited by self-emitted urine (Nevison et al., 2000). Our assay corroborates this known characteristic of countermarking behavior because a spot of nonself-urine (from C57BL/6J males) is able to promote robust countermarking from stimulus-naive test males of the BALB/c strain, whereas a spot of self-emitted (BALB/c) urine generates a response similar to that evoked by water (Figures 1A and 1B; Figures S1A and S1B available online). This behavior is dependent on social status because only dominant males mark in response to nonself-male cues (Figure S1A). Males also mark to female urine, though this behavior is evoked regardless of social status of the receiving male (Figure S1A). Marking behavior was not simply the result of environmental novelty because marking was not enhanced by the presence of the attractive odorant eugenol or the repulsive odor of ethanol (Figure S1C) (Logan et al., 2012). Females and castrated males did not show marking behavior in our assay (Figures S1D and S1E) (Desjardins et al., 1973; Kimura and Hagiwara, 1985). These controls confirm the robustness and reliability of our olfactory-mediated assay to investigate the role of olfactory cues in the release of countermarking behavior.

To isolate the male chemosignal(s) that promotes countermarking in our assay, we size fractionated the bioactive nonself (C57BL/6J)-urine and assayed countermarking from BALB/c males. Although distinctive volatile odors that vary between individuals compose the small molecule-containing low molecular weight (LMW) fraction, we found that this fraction lacked
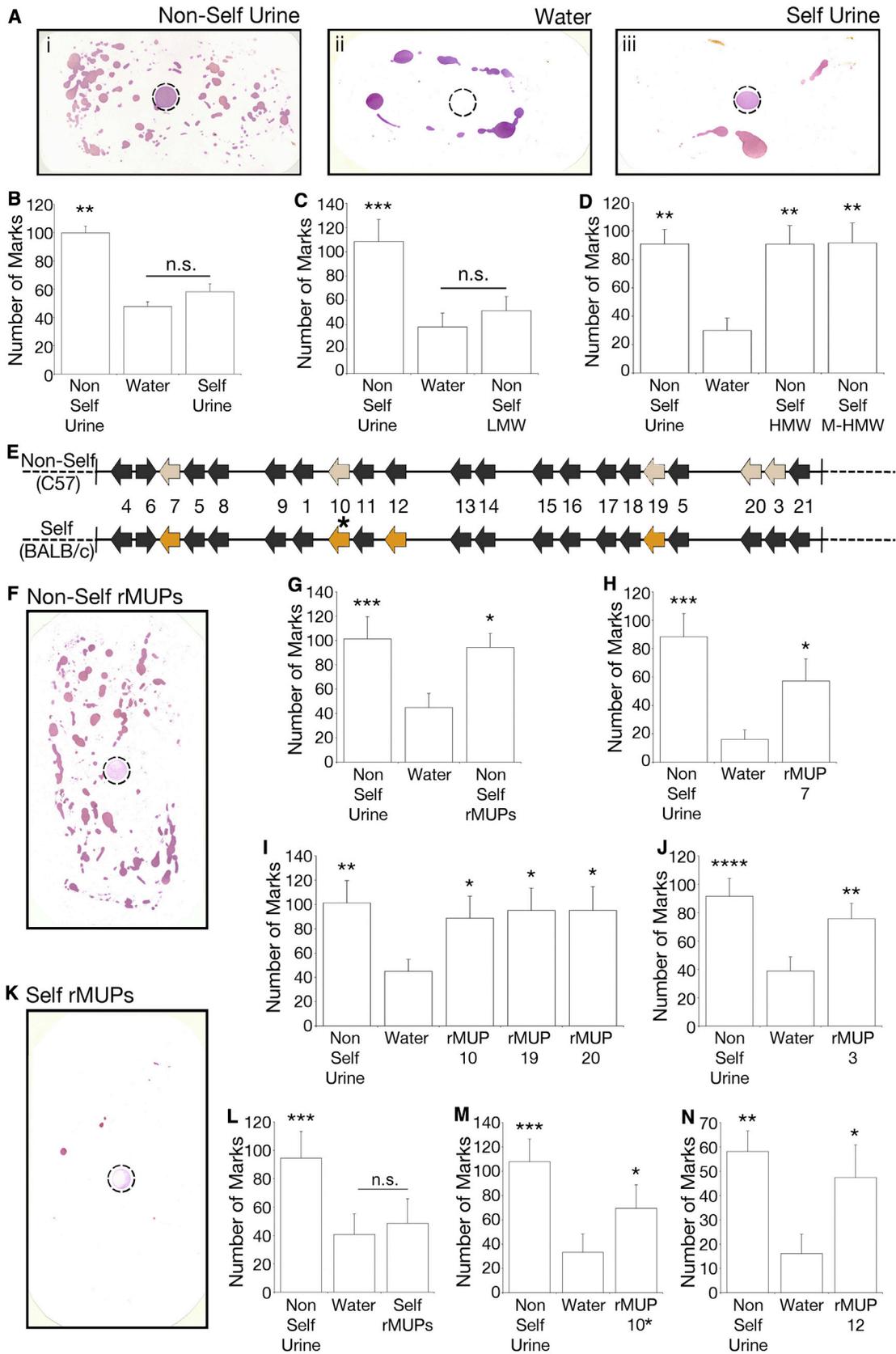
(legend on next page)

significant bioactivity. Instead, consistent with previous reports, males displayed a robust increase in marking behavior specifically toward the MUP-containing high molecular weight (HMW) fraction (Figures 1C and 1D) (Hurst et al., 2001; Nevison et al., 2003). MUPs are a large family of environmentally stable proteins that fold into a β-barrel structure that binds small hydrophobic molecules (Flower, 1996). Although MUPs have been previously implicated in promoting countermarking behavior, their precise role has not been determined (Hurst and Beynon, 2004). It has been postulated that they may act indirectly, perhaps serving to stabilize small molecules in the environment or transporting them into the mucous-filled VNO (Beynon and Hurst, 2003, 2004; Humphries et al., 1999; Hurst and Beynon, 2004; Hurst et al., 1998, 2001; Nevison et al., 2003; Novotny, 2003). However, when MUP-bound small molecules were competitively displaced by incubation of the HMW fraction with behaviorally inert menadione (Figure S1F) (Chamero et al., 2007; Xia et al., 2006) prior to assaying for behavior, we found that the countermarking-promoting bioactivity was retained, indicating that these small molecules do not instruct countermarking behavior (M-HMW; Figure 1D).

Of the 21 *Mups* encoded in the genome (Logan et al., 2008; Mudge et al., 2008), wild-caught individuals stably express variable subsets of 4–12 MUPs in their urine (Hurst and Beynon, 2004). The MUP expression of lab strains is genetically fixed so that all members of the same strain express identical MUPs, and some strains express a different, but fixed, MUP repertoire (Cheetham et al., 2009; Robertson et al., 1997). Although the overall amount of excreted protein can vary with social experience, the ratios of MUPs expressed are thought to be stable (Janotova and Stopka, 2011). Like major histocompatibility complex (MHC) peptides, MUPs display the hallmarks of individuality cues; however, such a function has only been tested in the presence of urinary small molecules, therefore, the significance of such customized protein excretion remains unknown (Hurst and Beynon, 2004; Hurst et al., 2001; Leinders-Zufall et al., 2004). We have previously isolated and cloned the MUP repertoires from both the C57BL/6J and BALB/cByJ lab strains (Figures 1E and S1G) (Chamero et al., 2007; Logan et al., 2008; Mudge et al., 2008; Robertson et al., 1997). A spot containing a mixture of bacterially expressed rMUPs corresponding to the five native MUPs excreted in nonself-C57BL/6J urine resulted in increased marking behavior from BALB/c males, indistinguish-

able from the response elicited by native urine (Figures 1F, 1G, and S1H). MUPs are detected by TRPC2-expressing VSNs (Chamero et al., 2007); therefore, we tested the marking response of TRPC2-deficient BALB/c males in our behavioral assay. We found that whereas these mutants marked in response to female cues, they did not display marking behavior to nonself-male cues (Figure S1I). This indicates that there are at least two different ligands that promote marking behavior: a male-emitted cue that is detected by TRPC2-expressing neurons, and a female-emitted cue that is TRPC2 independent. Together, these experiments indicate that the MUPs, devoid of their small molecule urinary ligands, serve as the male-emitted sensory cue that is sufficient to initiate countermarking behavior.

Previously, MUP20, also known as darcin, has been shown to be attractive to females and generate a conditioned place preference, whereas other MUPs do not share this bioactivity (Roberts et al., 2010, 2012). To determine if one particular MUP from the nonself-mixture is inherently dedicated to generate countermarking, individual rMUPs were singly assayed. We found no rMUP to be uniquely specialized to promote countermarking. Instead, each rMUP from the nonself-repertoire is equally sufficient to elicit a robust marking response compared to water (Figures 1H–1J). However, a blend of the four rMUPs corresponding to those excreted in self-urine was devoid of countermarking bioactivity, which is consistent with the response to self-emitted urine (Figures 1K and 1L). Surprisingly, such lack of activity is not due to an intrinsic difference between self- and nonself-MUPs because individual self-rMUPs assayed alone were each sufficient to generate increased countermarking compared to the control (Figures 1M and 1N, also Figures 1H and 1I). Moreover, the ability of individual rMUPs that compose the self-emitted MUP repertoire to elicit countermarking indicates that, despite constant exposure to their own urine, the failure of mice to display countermarking behavior to self-emitted cues is not due to sensory habituation. To evaluate whether orthologous MUPs similarly initiate countermarking, we tested a recombinant-expressed cat MUP, FelD4, which promotes fear behavior in mice (Papes et al., 2010). We found that mice did not countermark to this rMUP (Figure S1J). Together, our data reveal that individual mouse MUPs are each equally sufficient to promote countermarking; however, the repertoire of self-emitted MUPs fails to generate behavior.

**Figure 1. MUPs Are Sufficient to Elicit Marking Behavior**

(A) Representative blots of urine marks deposited by BALB/c males in response to olfactory stimuli (dotted circle): nonself (C57BL/6J)-urine (i), water (ii), or self (BALB/cByJ)-urine (iii).

(B–D) Quantification of urine marks to total urine (B), LMW fraction of nonself-urine (C), or HMW fraction with (HMW) or without (M-HMW) bound small molecules (D).

(E) Genomic representation of *Mup* gene cluster. Colored arrows indicate genes expressed by nonself (C57BL/6J, in top panel)- or self (BALB/cByJ, in bottom)-strains. The asterisk (*) indicates the Q159K MUP10 allelic variant present in BALB/cByJ; all other MUPs have the same amino acid sequences between the two strains.

(F and G) Behavioral response to a mixture of nonself-rMUPs (rMUP3+rMUP7+rMUP10+rMUP19+rMUP20).

(H–J) Quantification of behavioral responses to individual nonself-rMUPs.

(K and L) Behavioral response to a mixture of self-emitted rMUPs (rMUP7+rMUP10*+rMUP12+rMUP19; K and L) and to individual self-emitted rMUPs (M and N) (also see H and I) (n = 8–16).

Mean + SEM. p values were determined by a repeated-measures one-way ANOVA, with a Greenhouse-Geisser correction, followed by Bonferroni multiple comparisons test or by Friedman's nonparametric test followed by Dunn's multiple comparison test. ****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05; n.s., nonsignificant. p values were determined by comparison to water. See also Figure S1.
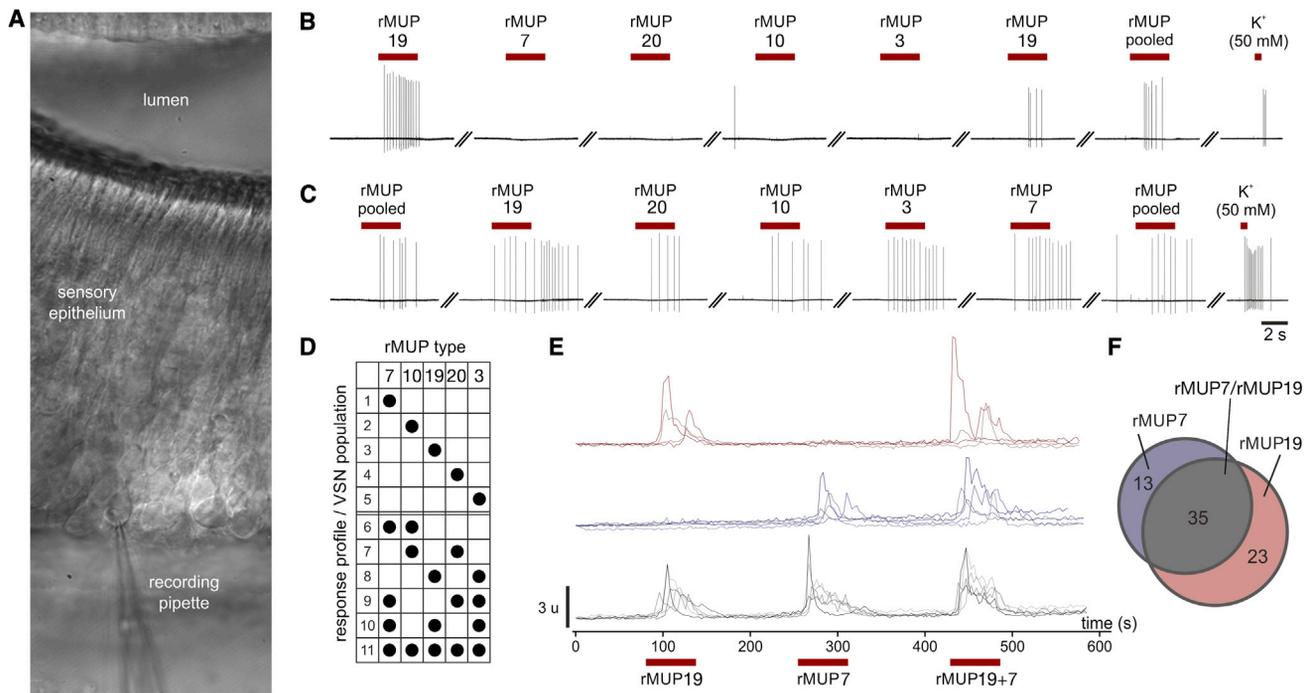
**Figure 2. VSNs Utilize a Combinatorial Code to Detect MUPs**

(A) VNO slice preparation to analyze individual sensory neurons showing the location of the recording pipette in the basal layer.

(B and C) Original "loose-seal" traces from two representative VSNs repetitively responding to a single (B) or all (C) rMUPs. Red bars indicate stimulation. Stimuli were applied in random order, interstimulus interval (ISI) = 60. Recordings are representative of VSN population #3 and #11, respectively, in (D).

(D) Summary of 11 distinct populations of neurons observed during extracellular recordings.

(E) Representative $Ca^{2+}$ transients imaged from dissociated VSNs sequentially stimulated with rMUP7 and rMUP19, which only differ by two amino acids (F56V and E140K), followed by a mixture of both MUPs. Colors indicate three distinct populations of VSNs based on response profile. Black bars indicate stimulus application. A total of 3,767 cells were imaged, sequentially exposed to all stimuli. 3 u, 3 × (F340/380 nm).

(F) Venn diagram quantifying the three distinct populations of neurons observed to respond to the two MUP stimuli by $Ca^{2+}$ imaging; colors correspond to VSN population in (E).

See also Figure S2.

## VSNs Detect MUPs Combinatorially

To function as an effective social cue, the signals that indicate self and other should be readily discernible by the receiver. The protein sequence of individual mouse MUPs can be up to 99% identical (Logan et al., 2008; Mudge et al., 2008). To determine the extent to which MUPs are differentially sensed, we recorded spike activity from basal VSNs in VNO slice preparations in response to repetitive, randomized stimulation with individual rMUPs (Hagendorf et al., 2009) (Figure 2A). We found 25 out of 1,006 basal neurons (2.5%) that responded to at least 1 single rMUP. Of these neurons, 52% (13 out of 25) were selectively activated by 1 single rMUP ligand (Figures 2B and S2A), a response pattern consistent with the highly tuned pheromone-detection properties previously observed in VSNs (Haga et al., 2010; Leinders-Zufall et al., 2000; Nodari et al., 2008). Unexpectedly, each rMUP activates at least three to four additional VSN populations (Figures 2C, 2D, and S2B–S2D). Some VSNs respond to specific combinations of few MUPs (5 out of 25 neurons; 20%) (Figures 2D, S2B, and S2D), whereas others are broadly tuned "generalists" that detect every rMUP tested (7 out of 25 neurons; 28%) (Figures 2C, S2C, and S2D). Together, the five rMUP ligands activate at least 11 VSN populations, and the variation

in VSN tuning enables individual rMUPs to be recognized by a unique combination of VSNs (Figures 2D and S2D). We additionally used a higher-throughput $Ca^{2+}$-imaging approach to analyze single dissociated VSNs (Chamero et al., 2007; Kaur et al., 2013) and similarly found individual rMUPs to stimulate both broadly and narrowly tuned neurons (Figures 2E, 2F, and S2E). Both methods of analysis indicate that each MUP activates multiple VSN types, and one VSN can detect multiple MUPs. This sensory strategy differs from the concept of highly tuned neurons that have been found to detect known mouse pheromones (Haga et al., 2010; Leinders-Zufall et al., 2000; Nodari et al., 2008) and, at least in part, appears to mirror the combinatorial-coding strategy employed by canonical olfactory neurons in the main olfactory epithelium (Malnic et al., 1999).

## VSNs Detect Relative Ratios of MUP Ligands

Another hallmark of MUP ligand expression is that individual MUPs are emitted at different ratios between different inbred strains, or among wild-caught individuals (although the ratio appears to be fixed in an individual). Therefore, the same MUP may be expressed at different concentrations between wild individuals (Cheetham et al., 2009; Robertson et al., 1997). Whether
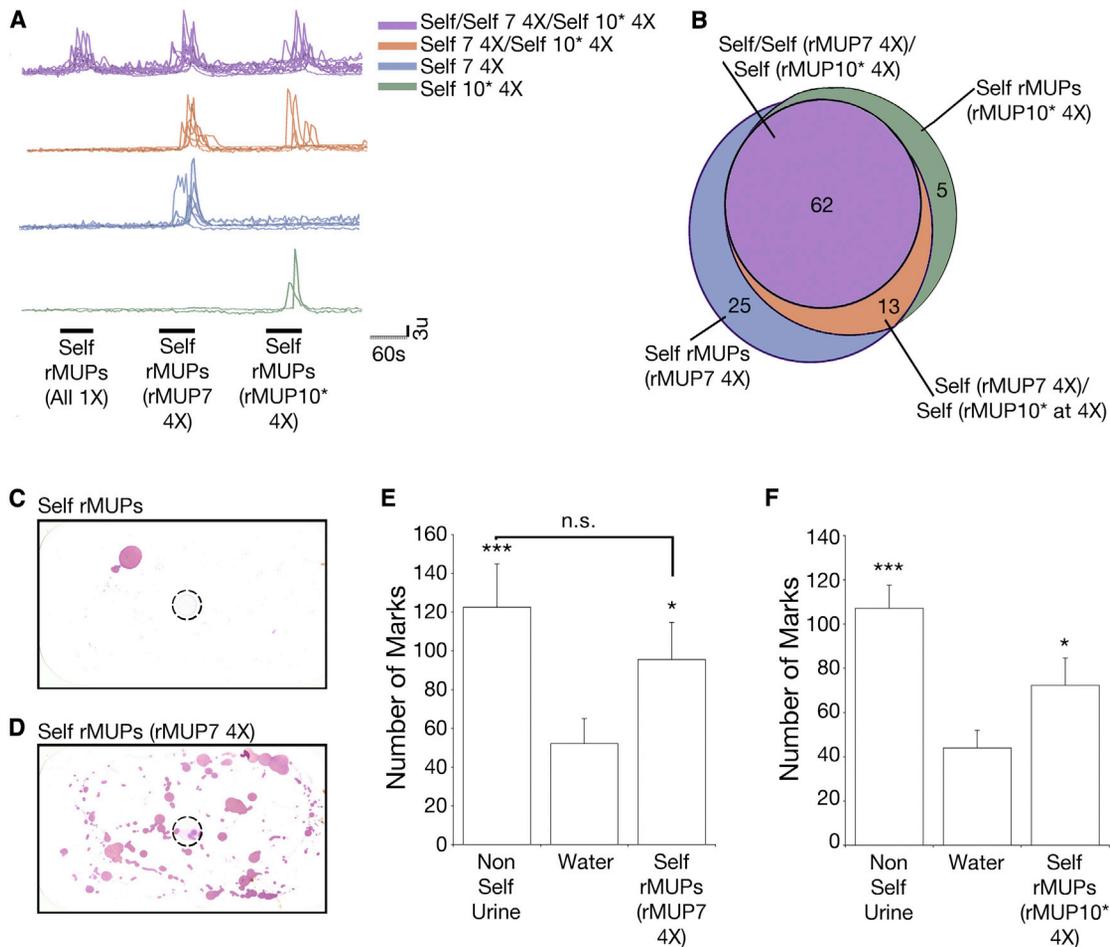
**Figure 3. Combinatorial Code Enables VSNs to Detect Relative Ratios of MUPs**

(A and B) Representative Ca$^{2+}$ transients from VSNs (A), quantified in (B). A total of 2,515 cells were imaged, sequentially exposed to self-rMUPs, self-rMUPs with rMUP7 at 4×, and self-rMUPs with rMUP10* at 4×. Colors indicate four distinct populations of VSNs based on response profile. Black bars indicate stimulus application. 3 u, 3 × (F340/380 nm).

(C and D) Countermarking behavior in response to a spot (dotted circle) of self-rMUPs (C), or self-rMUPs where the ratio of an individual MUP (rMUP7) was increased 4-fold (4×) (D).

(E and F) Quantification of behavioral response to self-rMUPs where the ratio of an individual MUP, rMUP7 (E), or rMUP10* (F) was increased 4-fold (n = 12). Mean + SEM is shown. p values were determined by a repeated-measures one-way ANOVA, with a Greenhouse-Geisser correction, followed by Bonferroni multiple comparisons test or by Friedman's test followed by Dunn's multiple comparison test. ****p < 0.0001; ***p < 0.001; ** p <0.01; *p<0.05; n.s., nonsignificant. p values were determined by comparison to water.

See also Figure S3.

differences in concentration are detected by males and used to expand the complexity of sensory information coding for behavior is not known. We used Ca$^{2+}$ imaging to assay the dose response of VSNs to three different rMUPs and found each to elicit a unique effective concentration and inflection point (Figure S3A). To determine if vomeronasal-response profiles could allow discrimination of relative MUP ratios, we compared the response of single neurons to the blend of self-rMUPs with all rMUPs at the same concentration, to an identical blend except the ratio of one rMUP component was increased 4-fold. We found that this change in rMUP ratio resulted in the stimulation of new VSN populations (Figures 3A and 3B). An increased ratio of rMUP7 activated an additional 38 neurons (1.5%), whereas altering the ratio of rMUP10* activated an additional

18 VSNs (0.7%). To determine if this concentration-dependent change in VSN response is functionally meaningful to the receiving male, we spotted the countermarking arena with the self-rMUP mixture in which the relative ratio of one rMUP was increased 4-fold. This change indeed endowed the previously inactive self-MUP repertoire with countermarking bioactivity (Figures 3C–3F). Our experiments indicate that the population of MUP-detecting VSNs can signal both the identity and the concentration of MUP ligands. Unlike the highly tuned response of other pheromone-detecting VSNs (Haga et al., 2010; Leinders-Zufall et al., 2000; Nodari et al., 2008), this alternate combinatorial-coding strategy can utilize fewer sensory receptors to transmit a large quantity of information about rapidly evolving and extremely similar ligands such as MUPs.

## Marking Behavior Depends on the Composition of the Detected MUP Repertoire

To investigate how self-emitted cues regulate behavior, we examined whether the blend of self-MUPs is sufficient to prevent countermarking. We assayed marking to the self-emitted rMUP repertoire that had been spiked with an additional unfamiliar rMUP (self-rMUPs +1) and found robust countermarking, indicating that the receiver is not solely utilizing the mere presence of self-MUPs to regulate marking behavior (Figures 4A and 4B). Imaging analysis of the VSN response to the self-MUPs +1 blend revealed the stimulation of additional VSNs that were not activated by the self-MUP blend (Figures 4C and S4A). In comparison, a single rMUP (equally capable of generating behavior; Figures 1H and 4D) only activated a subset of the neuron types stimulated by the full self-rMUP blend (Figure 4C). We further investigated the nature of the MUP code by removing just a single MUP from the self-rMUP blend (self-rMUPs −1) and found no countermarking behavior, similar to the response to self-rMUPs (Figures 4E and 4F). Correspondingly, the VSN activity evoked by both behaviorally inactive mixtures (self-rMUPs −1, and self-rMUPs) was indistinguishable (Figure 4G). However, when we produced a more dramatic alteration of the self-MUP blend by removing any combination of two of the four rMUPs (self-rMUPs −2), only a subset of the neurons activated by the self-MUPs was stimulated (Figures 4G and S4B), and detection of the self-rMUPs −2 mixtures again resulted in an increase in countermarking behavior compared to water (Figures 4H and S4C). These findings suggest that VSN activity is capable of resolving the identity of MUPs comprising a blend.

To analyze the extent to which the population of MUP-responsive VSNs can deviate from that activated by self-emitted MUPs to modulate the display of countermarking, we measured the behavioral response of individual males to 16 systematically manipulated rMUP mixtures and quantified the amount of marking behavior each blend induced (Figure S4D). We found that the extent of the behavioral response is increasingly altered as the rMUP mixture deviates from the self-emitted composition (Figure S4D). Next, we analyzed the VSN response to these same 16 MUP mixtures compared to behaviorally inactive self-rMUPs (Figures S4A, S4B, S4E–S4J, 3A, 3B, 4C, and 4G). We determined the percentage of individual neurons that responded to both manipulated rMUP and the self-rMUP stimuli to quantify the extent to which the identities of the responding VSNs matched the response to self-rMUPs. When we compared the percentage of animals displaying behavioral activity to the percentage of neural activity that was identical to the self-MUP-induced neural activity, we found a significant negative correlation (Spearman rank correlation coefficient of −0.79; p < 0.01; Figure 4I). A high percentage of overlap in neural activity of any stimulus compared to self-MUPs thus correlates with a low probability of countermarking behavior. In contrast, a low overlap in neural activity correlates with a high probability of countermarking.

Olfactory-mediated innate behavior in the mouse is known to be initiated by the detection of single salient ligands, and the surrounding odors in the environment have not been shown to modify their function (Dewan et al., 2013; Haga et al., 2010; Papes et al., 2010; Roberts et al., 2010, 2012). In contrast, whereas we do find that each MUP is sufficient to initiate behavior, when confronted with a complex blend of MUPs, the receiver initiates behavior based on the identity and concentration of the entire ensemble of detected MUPs. This signal can be rendered inert if it matches the self-emitted MUP repertoire.

## Significance of Self-MUP Repertoire Requires Experience

Because wild individuals, or individuals from different lab strains, each express a unique repertoire of MUPs, the ability of one's emitted MUP repertoire to modulate countermarking may be due to a correspondingly individualized, genetically encoded neural response to MUPs. Alternatively, the ability of one's own cues to modify behavior could be a result of each individual's unique sensory experience. To determine if sensory experience with MUPs can modulate countermarking, we aimed to artificially alter the MUP experience of test males with their native MUPs, which we found to be expressed after 4 weeks of age (Figure S5A). Therefore, we chronically exposed them to an additional MUP that is not part of the BALB/c-emitted MUP repertoire (MUP4) starting at 3 weeks of age. We assayed behavior upon maturity, at 8 weeks of age, and indeed found artificial MUP exposure to cause altered behavior. Subjects that experienced an additional rMUP displayed robust marking behavior to native self-urine (Figures 5A–5C).

We next sought to determine the extent to which odor experience could alter the significance of an established self-MUP repertoire in adult males. We first confirmed that self-emitted cues failed to increase marking behavior (Figure 5D). Next, we artificially exposed each subject to an alternate MUP repertoire by housing them in cages that had previously contained adult C57BL/6J males. This "artificial exposure" continuously provided a nonself-MUP repertoire experience. Individuals were assayed each week for their marking response to self- and nonself-urine. After an average of 2 weeks, we observed an increase in marking behavior to native self-urine, compared to water, in the majority of individuals (Figures 5E and S5B). The animals did not correspondingly decrease their response to nonself (C57BL/6J)-urine, likely because the artificial exposure contained a mixture of both C57BL/6J as well as self-emitted BALB/c MUPs. Finally, to determine if we could observe further experience-driven plasticity, we ensured that males were once again only exposed to self-emitted cues by maintaining them in clean cages. We found the response to self-emitted urine to revert to the original bioactivity: no different from the response to water (Figures 5F and S5B). The ability to repeatedly manipulate the countermarking response to native self-urine indicates that the self-MUP repertoire is not inherently restricted to attenuate countermarking of the emitting male. Rather, it is an individual's experience with the MUPs in his immediate environment that defines a unique signature of self that can be used to appropriately direct marking behavior.

## A Subset of Countermarking MUPs Additionally Promotes Male-Male Aggression

Currently, known mouse pheromones have only been implicated to promote a single function (Haga et al., 2010; Kimoto et al.,
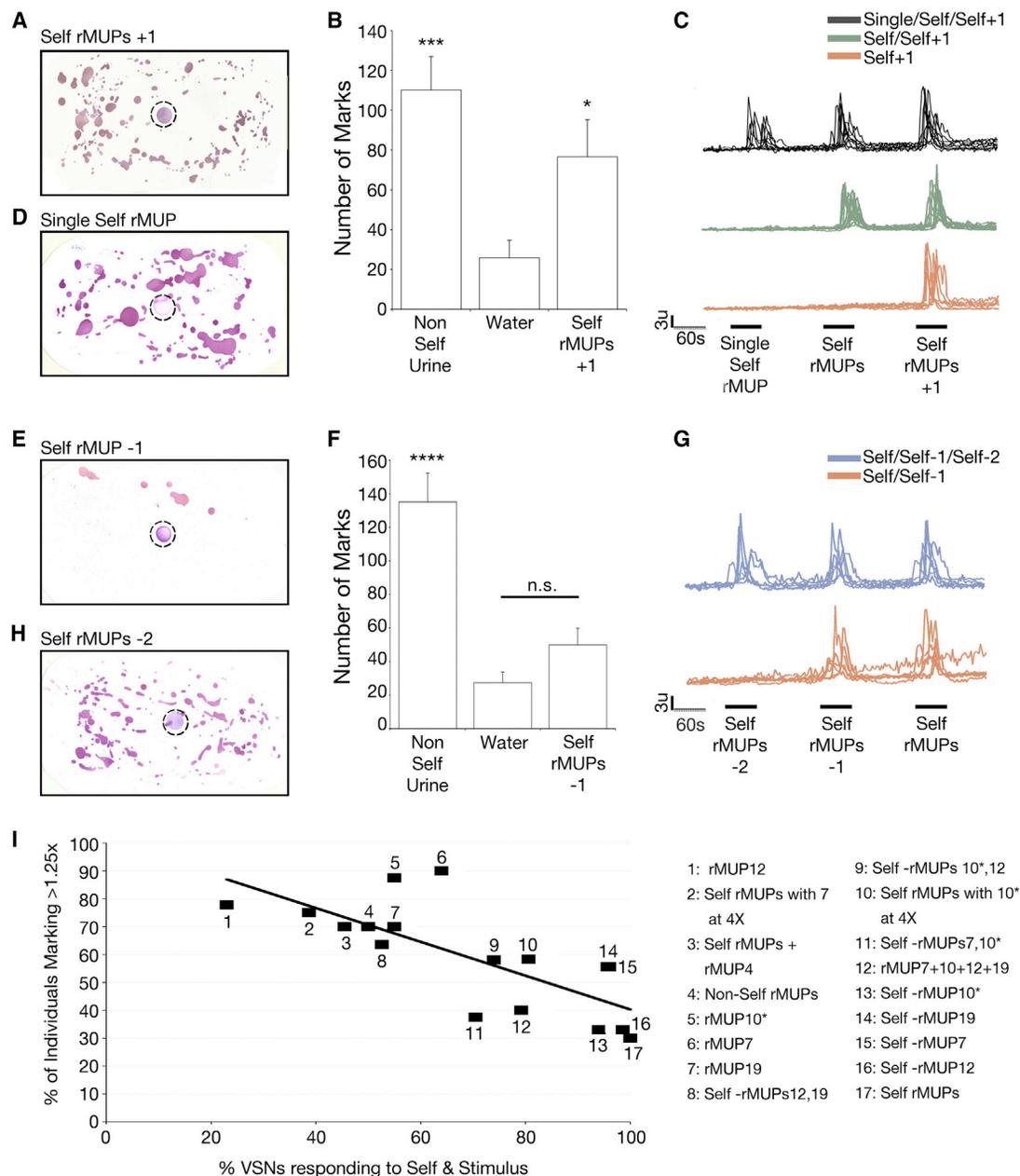
**Figure 4. The Identity and Ratio of Detected MUP Ensemble Modulate Behavior**

(A) Countermarking behavior in response to a spot (dotted circle) of self-rMUPs plus the additional rMUP4 (self-rMUPs +1).

(B) Quantification of behavioral response to self-rMUPs plus the additional rMUP4 (self-rMUPs +1) (n = 12).

(C) Representative $Ca^{2+}$ transients from VSNs. A total of 2,200 cells were imaged, sequentially exposed to rMUP7, self-rMUPs, and self-rMUP+rMUP4. Colors indicate three distinct populations of VSNs based on response profile. 3 u, 3 × (F340/380 nm).

(D) Countermarking behavior in response to a spot (dotted circle) of a single self-rMUP (rMUP7).

(E and F) Countermarking behavior in response to a spot of self-rMUPs −1 (self-rMUPs without rMUP 7). (F) Summed marking response is shown to every combination of three of the four self-rMUPs (self-rMUPs −1) (n = 36).

(G) Representative $Ca^{2+}$ transients from VSNs. A total of 3,893 cells were imaged, sequentially exposed to self-rMUPs −2, self-rMUPs −1, and self-rMUPs. Colors indicate two distinct populations of VSNs based on response profile. Black bars indicate stimulus application. 3 u, 3 × (F340/380 nm).

(H) Countermarking behavior in response to a spot of self-rMUPs −2 (self-rMUPs without rMUP10* and rMUP12).

(I) An inverse relationship exists between countermarking behavior and the percentage of VSNs that respond both to the indicated stimulus and to self-rMUPs. Best linear fit, $R^2 = 0.56$.

Mean + SEM is shown. p values were determined by a repeated-measures one-way ANOVA, with a Greenhouse-Geisser correction, followed by Bonferroni multiple comparisons test or by Friedman's test followed by Dunn's multiple comparison test. ****$p < 0.0001$; ***$p < 0.001$; ** $p < 0.01$; *$p < 0.05$; n.s., nonsignificant. p values were determined by comparison to water. See also Figure S4.
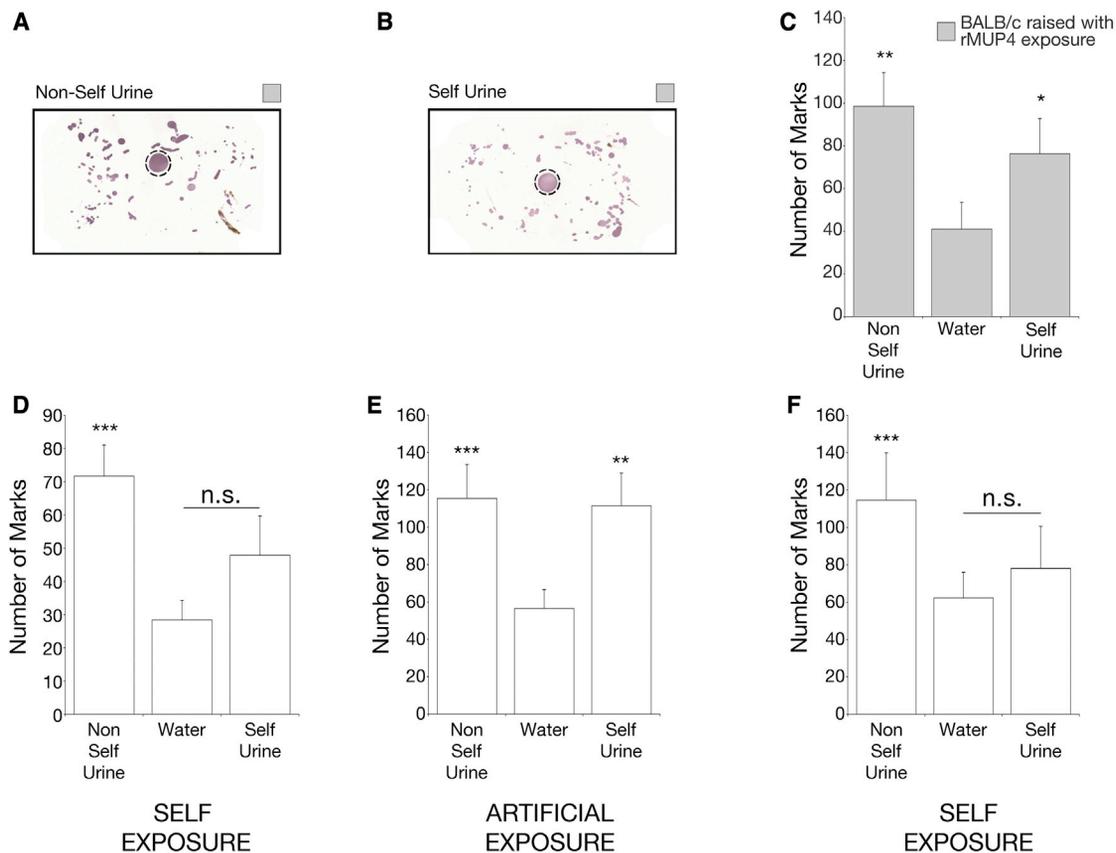
**Figure 5. Signature of "Self" Is Based on Experience**

(A and B) Countermarking behavior of males raised with rMUP4 following exposure to a spot (dotted circle) of nonself-urine (A) or self (BALB/c)-urine (B).

(C) Behavioral response of males raised with rMUP4 exposure shows marking to self (BALB/c)-urine (n = 11).

(D–F) Behavioral response of adult males changes with odor experience. Males were raised among self-odors (D), then exposed to nonself-male soiled bedding (E), and returned to self-odor environment (F) (n = 7–15).

p values were determined by Friedman's test followed by Dunn's multiple comparison test. ***p < 0.001; **p < 0.01; *p < 0.05; n.s., nonsignificant. p values were determined by comparison to water.

See also Figure S5.

2005; Roberts et al., 2010). We have previously shown that the pool of C57BL/6J rMUPs elicits male-male territorial aggression (Chamero et al., 2007). To investigate how MUPs promote multiple behavioral outputs, we assayed rMUPs as stimuli in a resident-intruder assay using C57BL/6J male subjects (Figure 6A) (Chamero et al., 2007). Unlike countermarking, we found that the pool of self-rMUPs is not inert; it contains significant aggression-promoting bioactivity (Figure 6A). Moreover, when individually assayed, we found that not all rMUPs promote aggression. Three rMUPs were unable to generate any detectible behavior, whereas two proteins, rMUP20 and rMUP3, were each sufficient to release the full fixed action pattern of aggression (Figure 6A). To determine if a change in MUP concentration alters its likelihood to promote aggression, we tested the function of rMUPs at 1× and 4× concentrations. Even when present at high concentrations, three MUPs failed to promote the behavior; only rMUP20 and rMUP3 increase the aggressive behavior of males (Figure 6B). Together, these results indicate that, unlike countermarking that utilizes the full repertoire of

detected MUPs to initiate behavior, MUP3 and MUP20 promote aggressive behavior regardless of the urine odor milieu, repertoire of MUPs, or MUP concentration.

**MUP3 Triggers Experience-Independent Innate Aggression in a Context-Dependent Manner**

Because the countermarking response to self-MUPs requires MUP experience (Figure 5), we aimed to test the extent to which ligand experience during development influences its capacity to generate aggressive behavior in adults. Although all males of the C57BL/6J lab strain express both aggression-promoting MUPs, mass spectrometry analysis shows that BALB/cByJ males do not express either MUP20 or MUP3 (Figure 1E) (Cheetham et al., 2009; Logan et al., 2008). To determine whether BALB/c males express any alternate aggression-promoting MUP(s), we tested the bioactivity of the MUP-containing HMW urine fraction from the BALB/c strain in the aggression assay and found no aggression-promoting bioactivity (Figures 7A and S6). This confirms that BALB/c
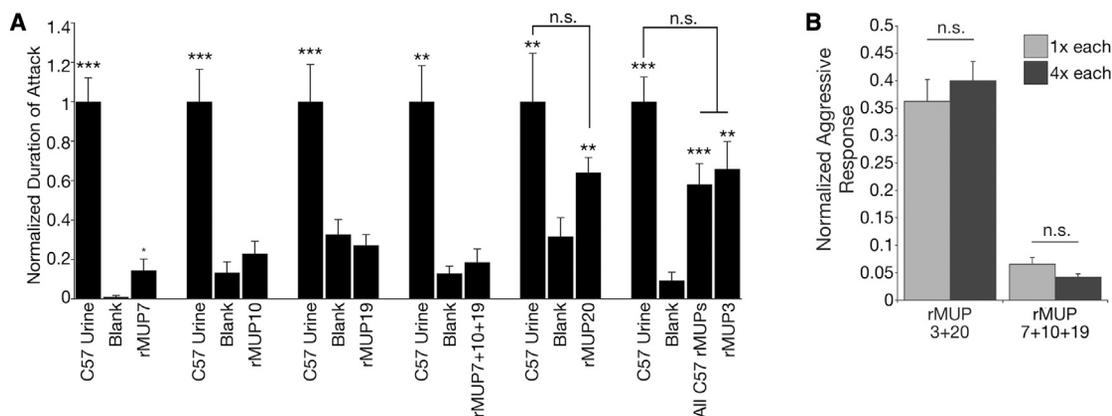
**Figure 6. Only a Subset of MUPs Promotes Aggression**
(A) Only a subset of rMUPs swabbed on castrate mice stimulates aggression in the resident-intruder assay. Black bars indicate C57 male residents (n = 6–51).
(B) Aggression-promoting bioactivity of MUPs is not sensitive to ligand concentration. Response to negative control was subtracted from response to positive control and test stimulus. Response to test stimulus was then normalized to the response to positive control in order to compute the values presented. 1×, 5 mg/ml of each protein in the mix; 4×, 20 mg/ml of each protein in the mix (n = 12).
Mean + SEM. p values were determined by Friedman's test followed by Dunn's multiple comparison test. ***p < 0.001; **p < 0.01; *p < 0.05; n.s., nonsignificant. p values were determined by comparison to water.

can serve as a natural loss-of-function strain in which males are experientially naive to aggression-promoting MUP. When presented with rMUP3 in the resident-intruder assay, we found that a BALB/c male's innate response is statistically indistinguishable from his aggressive response to the odor of whole urine (Figure 7B). These experiments indicate that rMUP3 is intrinsically specialized with a determined function, and its sensory detection activates hardwired neural circuits that generate innate aggression.

We next began to investigate how MUP3 and MUP20 regulate both aggression and countermarking. Of wild-caught males, and most inbred lab strains, 92% have been found to express an aggression-promoting MUP (Armstrong et al., 2005; Roberts et al., 2010). If MUP3 and MUP20 function to elicit aggression, and individuals are continuously expressing these signals, then most males would be constantly aroused to fight. However, we observe that singly housed males do not show arousal and motor patterns consistent with aggressive behavior. We confirmed this by exposing males to an aggression-promoting MUP, rMUP3, in the countermarking assay and quantified six variables of locomotion to determine if it elicits any motor patterns characteristic of aggression. We found the responses to rMUP3 to be indistinguishable from that of the nonaggression-promoting rMUP7, with neither showing any motor patterns of aggression (Figure 7C). This is consistent with observations by us and others that defining motor patterns of aggression (such as lunging, biting, chasing, cornering, and kicking) require a physical target on which to direct attacks (Blanchard et al., 2003; Choi et al., 2011; Stowers et al., 2013) (L.S., unpublished data), and indicates that the extended sensory environment contributes to MUP significance. Our data provide a framework to begin to investigate the mechanisms by which a single ligand promotes two entirely different motor outputs, with aggression being released only in the presence of a target animal.

## DISCUSSION

Very little is known about how the environment is transformed into a meaningful neural code that regulates behavior. Behavioral analysis of mice indicates that a subset of odor cues emitted and detected between members of the same species, termed pheromones, is specialized to generate social behavior (Karlson and Luscher, 1959; Wyatt, 2010). Because few of these specialized odors have been isolated and studied in mammals, there is little understanding of whether they function as originally proposed (Wyatt, 2010). The purification of ligands with a known output enables us to now gain insight into several basic qualities of the neural strategy that underlies sensory-evoked social behavior. First, we find that MUP3 is specialized in that it is intrinsically fixed to promote aggression upon first encounter. This reveals that a subset of the cognate sensory neurons is genetically hardwired to gain access to central neural circuits of aggression (Adams, 2006; Nelson and Trainor, 2007). Second, unlike currently known mouse pheromones (Haga et al., 2010; Roberts et al., 2010, 2012), we find that MUP3 is not sufficient to generate the motor patterns of aggression; instead, it requires additional, coincident, sensory information from a target animal (Blanchard et al., 2003; Choi et al., 2011) that may function as a gate to ensure that males do not become aggressive to their own signals. MUP3 and MUP20 can now be used as tools to activate, identify, and begin to study the neural circuits that generate aggression and the mechanisms that regulate the display of the behavior depending on the extended sensory context. Third, we also find aggression-promoting MUPs to be sufficient to evoke a second, entirely different social behavior: countermarking. Surprisingly, this indicates that subsets of VSNs have the capacity to elicit multiple outputs. It will be of great interest to determine how VSNs are organized and regulated to differentially activate multiple downstream circuits. The identification of MUP3 as a bioactive odor cue will provide an
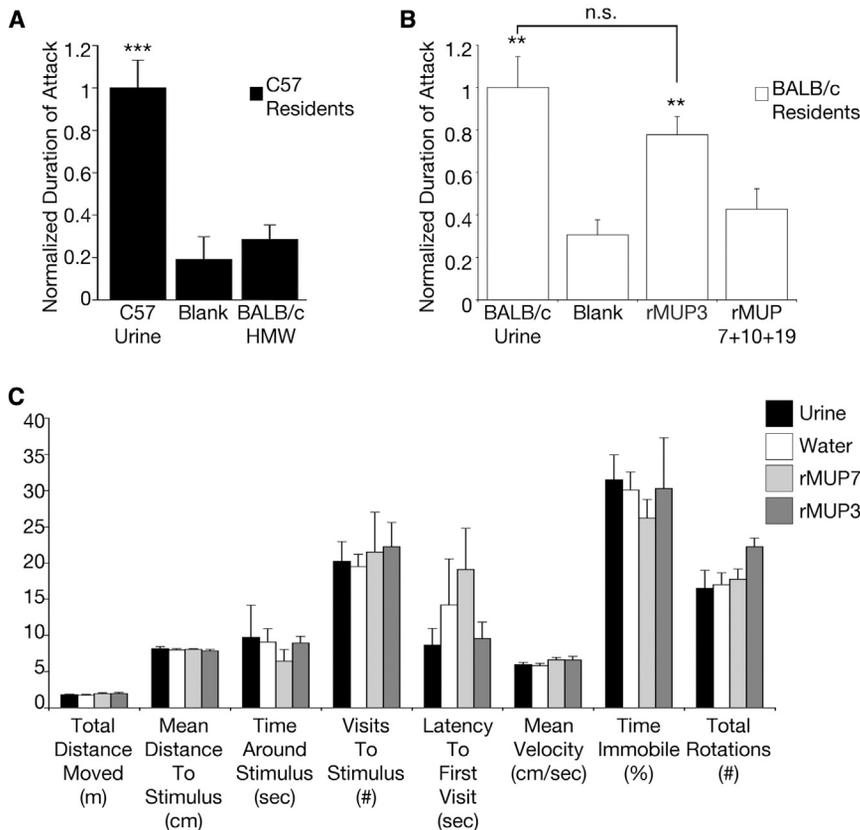
**Figure 7. MUPs Promote Innate Aggression**

(A) Native BALB/c HMW fraction does not promote aggression in the resident-intruder assay. Black bars indicate C57BL/6J male residents (n = 5–20).

(B) First detection of rMUP3 stimulates aggression in BALB/c males. White bars indicate BALB/c male residents (n = 12). Mean + SEM is shown. p values were determined by ANOVA followed by Tukey-honest significant difference (HSD) post hoc analysis or by Kruskal-Wallis test. ***p < 0.001; **p < 0.01; *p < 0.05; n.s., nonsignificant.

(C) Locomotor behavior of test animals during odor-mediated countermarking assay as measured by tracking software (n = 4). All values are mean + SEM. No significant differences were found by one-tailed ANOVA.

See also Figure S6.

essential tool to identify and study the neural circuit mechanisms that underlie the innate response, context dependency, and general circuit structure that generates aggressive behavior.

The MUP sensory code that generates countermarking is strikingly different from that of aggression or other known specialized odor cues (Haga et al., 2010; Papes et al., 2010; Roberts et al., 2010). Our behavioral analysis indicates that MUP-mediated countermarking is dependent on evaluating the entire repertoire of MUPs in the environment. This indicates that there is a neural mechanism to identify and compare all of the sensory activity generated by this ligand family. Second-order mitral cell neurons have been shown to monitor multiple glomeruli, of presumably closely related receptors (Wagner et al., 2006). This organization may serve to "read" coincident MUP activity directly in the accessory olfactory bulb, or it may occur through undefined mechanisms in higher-order processing centers. How the detection of multiple MUP ligands informs countermarking, but has no observed effect on aggression, remains to be studied.

Previously studied VSNs have been found to be tuned to a cognate ligand. Instead, we find that the subset of MUP-detecting neurons utilizes a combinatorial strategy. It has previously been noted that very few neurons in the VNO respond to male mouse odor sources (Isogai et al., 2011). Although this may indicate that other sensory neurons, perhaps in the main olfactory epithelium, primarily detect male odors, our data indicate an alternate strategy mediated within the VNO. Combinatorial cod-

ing enables a small subset of receptors to identify a large number of structurally similar ligands, provides for the detection of rapidly evolving protein ligands (such as species-specific MUPs), and is able to differentiate relative ratios of individual members thereby increasing their functional coding capacity. The extent to which MUP sensory neurons are unique among VSNs in utilizing combinatorial coding remains to be determined.

Using simplified laboratory assays, it is possible to control an individual's experience, internal state, and environment in order to robustly initiate and study social behavior. Although this approach is essential for first identification of the underlying general neural characteristics, the neural mechanisms and responses in the wild are likely to be dynamic, more complex, and less predictable. Even in a controlled environment, the probability of pheromones to initiate their cognate behavior depends on the gender, age, social status, and reproductive state of the receiver. Countermarking is an extremely simple behavior that has a high probability of occurring in the presence of urine and can serve as a model to study the neural mechanisms that enable and modify the release of behavior. Among mice, and across species, individuals have to decipher the origin of all detected biosignals, whether they are self-generated or emitted by others. Although this computation appears effortless, the underlying neural mechanisms remain largely unknown. Our identification of the relevant ligands that signal self and other for countermarking behavior enabled us to determine that the distinction is not due to sensory habituation. Instead, the countermarking action of MUPs can be modulated by previous experience, with the action of self-emitted MUPs rendered inert. This indicates that MUP sensory activity is likely to intersect with a memory of previous MUP experience to inhibit output countermarking behavior. The formation of olfactory memories and the role of experience to modify innate behavior have been difficult to study. The identification of bioactive ligands now provides a relatively simple experimental platform to determine how the olfactory template of the self-emitted

ligands is created, where in the brain it is stored, and how it inhibits the release of countermarking.

## EXPERIMENTAL PROCEDURES

### Animals and Behavioral Assays

Seven to 12-week-old C57BL/6J and BALB/cByJ males were maintained in groups of four to five animals per cage, except those used in behavioral paradigms, which were pair housed. Countermarking behavior was measured by placing test animals into individual clean Whatman paper-lined cages, with 50 μl stimulus pipetted onto the center, for 5 min. Collected Whatman paper was treated with ninhydrin solution, which binds proteins and turns purple upon baking, allowing visualization of urine marks. The blots were then digitally scanned to quantify the number of spots per sheet. All animal procedures were in compliance with institutional guidelines, Institutional Animal Care and Use Committee, European Union legislation (Directive 86/609/EEC), and FELASA recommendations.

### Stimuli

Urine was freshly gathered from wild-type mice for use in all experiments. rMUPs were prepared using the pMAL Protein Fusion and Purification System (Chamero et al., 2007). Each rMUP was presented at 5 mg/ml (1×) for behavioral assays and diluted 1:300 for $Ca^{2+}$ imaging. Wherever proteins were presented in a blend, each protein was present at 5 mg/ml (for a total of 10–25 mg/ml protein in the blend). Control stimuli were used at the following concentrations: 4 mg/ml menadione in ethanol, 20 mg/ml BSA, 70% ethanol in water, and 100 nM eugenol in water.

### $Ca^{2+}$ Imaging

Transient increase in free $Ca^{2+}$ concentration in dissociated VSNs was determined by ratiometric fura-2 fluorescence (Chamero et al., 2007). Experiments were limited to three stimuli to maintain viability of the neurons. Dissociated VSNs were sequentially exposed to all listed stimuli, and response profiles were scored. Neural responses were scored if they met all of the following criteria: (1) ≥1.5× increase in fluorescence ratio (over baseline signal) during stimulus presentation, (2) ≥1.5× increase in fluorescence ratio (over baseline signal) response to the positive control stimulus, and (3) less than 1.5× (over baseline signal) increase in fluorescence ratio outside the stimulus presentation window. See Extended Experimental Procedures for more information.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.02.025.

## REFERENCES

Adams, D.B. (2006). Brain mechanisms of aggressive behavior: an updated review. Neurosci. Biobehav. Rev. 30, 304–318.

Armstrong, S.D., Robertson, D.H., Cheetham, S.A., Hurst, J.L., and Beynon, R.J. (2005). Structural and functional differences in isoforms of mouse major urinary proteins: a male-specific protein that preferentially binds a male pheromone. Biochem. J. 391, 343–350.

Bacchini, A., Gaetani, E., and Cavaggioni, A. (1992). Pheromone binding proteins of the mouse, Mus musculus. Experientia 48, 419–421.

Beynon, R.J., and Hurst, J.L. (2003). Multiple roles of major urinary proteins in the house mouse, Mus domesticus. Biochem. Soc. Trans. 31, 142–146.

Beynon, R.J., and Hurst, J.L. (2004). Urinary proteins and the modulation of chemical scents in mice and rats. Peptides 25, 1553–1563.

Blanchard, R.J., Wall, P.M., and Blanchard, D.C. (2003). Problems in the study of rodent aggression. Horm. Behav. 44, 161–170.

Chamero, P., Marton, T.F., Logan, D.W., Flanagan, K., Cruz, J.R., Saghatelian, A., Cravatt, B.F., and Stowers, L. (2007). Identification of protein pheromones that promote aggressive behaviour. Nature 450, 899–902.

Chamero, P., Katsoulidou, V., Hendrix, P., Bufe, B., Roberts, R., Matsunami, H., Abramowitz, J., Birnbaumer, L., Zufall, F., and Leinders-Zufall, T. (2011). G protein G(alpha)o is essential for vomeronasal function and aggressive behavior in mice. Proc. Natl. Acad. Sci. USA 108, 12898–12903.

Cheetham, S.A., Smith, A.L., Armstrong, S.D., Beynon, R.J., and Hurst, J.L. (2009). Limited variation in the major urinary proteins of laboratory mice. Physiol. Behav. 96, 253–261.

Choi, G.B., Stettler, D.D., Kallman, B.R., Bhaskar, S.T., Fleischmann, A., and Axel, R. (2011). Driving opposing behaviors with ensembles of piriform neurons. Cell 146, 1004–1015.

Desjardins, C., Maruniak, J.A., and Bronson, F.H. (1973). Social rank in house mice: differentiation revealed by ultraviolet visualization of urinary marking patterns. Science 182, 939–941.

Dewan, A., Pacifico, R., Zhan, R., Rinberg, D., and Bozza, T. (2013). Nonredundant coding of aversive odours in the main olfactory pathway. Nature 497, 486–489.

Finlayson, J.S., Asofsky, R., Potter, M., and Runner, C.C. (1965). Major urinary protein complex of normal mice: origin. Science 149, 981–982.

Flower, D.R. (1996). The lipocalin protein family: structure and function. Biochem. J. 318, 1–14.

Haga, S., Hattori, T., Sato, T., Sato, K., Matsuda, S., Kobayakawa, R., Sakano, H., Yoshihara, Y., Kikusui, T., and Touhara, K. (2010). The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. Nature 466, 118–122.

Hagendorf, S., Fluegge, D., Engelhardt, C., and Spehr, M. (2009). Homeostatic control of sensory output in basal vomeronasal neurons: activity-dependent expression of ether-à-go-go-related gene potassium channels. J. Neurosci. 29, 206–221.

Hastie, N.D., Held, W.A., and Toole, J.J. (1979). Multiple genes coding for the androgen-regulated major urinary proteins of the mouse. Cell 17, 449–457.

Humphries, R.E., Robertson, D.H., Beynon, R.J., and Hurst, J.L. (1999). Unravelling the chemical basis of competitive scent marking in house mice. Anim. Behav. 58, 1177–1190.

Hurst, J.L., and Beynon, R.J. (2004). Scent wars: the chemobiology of competitive signalling in mice. BioEssays 26, 1288–1298.

Hurst, J.L., Robertson, D.H.L., Tolladay, U., and Beynon, R.J. (1998). Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. Anim. Behav. 55, 1289–1297.

Hurst, J.L., Payne, C.E., Nevison, C.M., Marie, A.D., Humphries, R.E., Robertson, D.H., Cavaggioni, A., and Beynon, R.J. (2001). Individual recognition in mice mediated by major urinary proteins. Nature 414, 631–634.

Isogai, Y., Si, S., Pont-Lezica, L., Tan, T., Kapoor, V., Murthy, V.N., and Dulac, C. (2011). Molecular organization of vomeronasal chemoreception. Nature 478, 241–245.

Janotova, K., and Stopka, P. (2011). The level of major urinary proteins is socially regulated in wild Mus musculus musculus. J. Chem. Ecol. 37, 647–656.

Karlson, P., and Luscher, M. (1959). Pheromones': a new term for a class of biologically active substances. Nature *183*, 55–56.

Kaur, A., Dey, S., and Stowers, L. (2013). Live cell calcium imaging of dissociated vormeronasal neurons. Methods Mol. Biol. *1068*, 189–200.

Kimoto, H., Haga, S., Sato, K., and Touhara, K. (2005). Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. Nature *437*, 898–901.

Kimura, T., and Hagiwara, Y. (1985). Regulation of urine marking in male and female mice: effects of sex steroids. Horm. Behav. *19*, 64–70.

Knopf, J.L., Gallagher, J.F., and Held, W.A. (1983). Differential, multihormonal regulation of the mouse major urinary protein gene family in the liver. Mol. Cell. Biol. *3*, 2232–2240.

Leinders-Zufall, T., Lane, A.P., Puche, A.C., Ma, W., Novotny, M.V., Shipley, M.T., and Zufall, F. (2000). Ultrasensitive pheromone detection by mammalian vomeronasal neurons. Nature *405*, 792–796.

Leinders-Zufall, T., Brennan, P., Widmayer, P., S, P.C., Maul-Pavicic, A., Jäger, M., Li, X.H., Breer, H., Zufall, F., and Boehm, T. (2004). MHC class I peptides as chemosensory signals in the vomeronasal organ. Science *306*, 1033–1037.

Logan, D.W., Marton, T.F., and Stowers, L. (2008). Species specificity in major urinary proteins by parallel evolution. PLoS One *3*, e3280.

Logan, D.W., Brunet, L.J., Webb, W.R., Cutforth, T., Ngai, J., and Stowers, L. (2012). Learned recognition of maternal signature odors mediates the first suckling episode in mice. Curr. Biol. *22*, 1998–2007.

Malnic, B., Hirono, J., Sato, T., and Buck, L.B. (1999). Combinatorial receptor codes for odors. Cell *96*, 713–723.

Meredith, M., and O'Connell, R.J. (1979). Efferent control of stimulus access to the hamster vomeronasal organ. J. Physiol. *286*, 301–316.

Mudge, J.M., Armstrong, S.D., McLaren, K., Beynon, R.J., Hurst, J.L., Nicholson, C., Robertson, D.H., Wilming, L.G., and Harrow, J.L. (2008). Dynamic instability of the major urinary protein gene family revealed by genomic and phenotypic comparisons between C57 and 129 strain mice. Genome Biol. *9*, R91.

Nelson, R.J., and Trainor, B.C. (2007). Neural mechanisms of aggression. Nat. Rev. Neurosci. *8*, 536–546.

Nevison, C.M., Barnard, C.J., Beynon, R.J., and Hurst, J.L. (2000). The consequences of inbreeding for recognizing competitors. Proc. Biol. Sci. *267*, 687–694.

Nevison, C.M., Armstrong, S., Beynon, R.J., Humphries, R.E., and Hurst, J.L. (2003). The ownership signature in mouse scent marks is involatile. Proc. Biol. Sci. *270*, 1957–1963.

Nodari, F., Hsu, F.F., Fu, X., Holekamp, T.F., Kao, L.F., Turk, J., and Holy, T.E. (2008). Sulfated steroids as natural ligands of mouse pheromone-sensing neurons. J. Neurosci. *28*, 6407–6418.

Novotny, M.V. (2003). Pheromones, binding proteins and receptor responses in rodents. Biochem. Soc. Trans. *31*, 117–122.

Novotny, M.V., Ma, W., Wiesler, D., and Zídek, L. (1999). Positive identification of the puberty-accelerating pheromone of the house mouse: the volatile ligands associating with the major urinary protein. Proc. Biol. Sci. *266*, 2017–2022.

Papes, F., Logan, D.W., and Stowers, L. (2010). The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. Cell *141*, 692–703.

Rich, T.J., and Hurst, J.L. (1999). The competing countermarks hypothesis: reliable assessment of competitive ability by potential mates. Anim. Behav. *58*, 1027–1037.

Roberts, S.A., Simpson, D.M., Armstrong, S.D., Davidson, A.J., Robertson, D.H., McLean, L., Beynon, R.J., and Hurst, J.L. (2010). Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. BMC Biol. *8*, 75.

Roberts, S.A., Davidson, A.J., McLean, L., Beynon, R.J., and Hurst, J.L. (2012). Pheromonal induction of spatial learning in mice. Science *338*, 1462–1465.

Robertson, D.H., Hurst, J.L., Bolgar, M.S., Gaskell, S.J., and Beynon, R.J. (1997). Molecular heterogeneity of urinary proteins in wild house mouse populations. Rapid Commun. Mass Spectrom. *11*, 786–790.

Stowers, L., Cameron, P., and Keller, J.A. (2013). Ominous odors: olfactory control of instinctive fear and aggression in mice. Curr. Opin. Neurobiol. *23*, 339–345.

Szoka, P.R., and Paigen, K. (1978). Regulation of mouse major urinary protein production by the Mup-A gene. Genetics *90*, 597–612.

Wagner, S., Gresser, A.L., Torello, A.T., and Dulac, C. (2006). A multireceptor genetic approach uncovers an ordered integration of VNO sensory inputs in the accessory olfactory bulb. Neuron *50*, 697–709.

Wyatt, T.D. (2010). Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. *196*, 685–700.

Xia, J., Sellers, L.A., Oxley, D., Smith, T., Emson, P., and Keverne, E.B. (2006). Urinary pheromones promote ERK/Akt phosphorylation, regeneration and survival of vomeronasal (V2R) neurons. Eur. J. Neurosci. *24*, 3333–3342.

# Supplemental Information

## EXTENDED EXPERIMENTAL PROCEDURES

### Animals

7-12 week old C57BL/6J and BALB/cByJ males were maintained in groups of 4-5 animals per cage, except those used in behavioral paradigms, which were pair housed. For control experiments, wild-type BALB/cByJ males were surgically castrated at 3 weeks of age and allowed to mature to 8 weeks of age. 8-12 week old BALB/cByJ females and castrated males were singly housed for 10 days prior to behavior assays. TRPC2$^{-/-}$ mice were obtained from heterozygous mating couples that had previously been fully back-crossed into the BALB/cByJ background (Flanagan et al., 2011). Males were housed singly before behavioral testing. All animal procedures were in compliance with institutional guidelines, Institutional Animal Care and Use Committee, European Union legislation (Directive 86/609/EEC) and FELASA recommendations.

### Stimuli

Wild-type mice were used as the source of urine in all experiments. Self urine was gathered from group housed 8-12 week old BALB/c males (not the test male) and non-self urine was gathered from group housed 8-12 week old C57Bl/6J males. Urine was gathered fresh daily by holding mice by the scruff of their necks over a sterile zip lock bag. Deposited urine was collected with a transfer pipette. Urine from at least 2-3 mice was pooled for each stimulus, and was used without dilution. 0.5-1 ml of urine was size fractionated by centrifugation and resuspended to initial volume (Chamero et al., 2007). Menadione displacement was performed as previously described (Chamero et al., 2007). 50 $\mu$l of 4mg/ml menadione in ethanol, 20 mg/ml BSA, 70% ethanol in water, and 100 nM eugenol in water were used as control stimuli in behavioral assays.

### Production of rMUPs

Full-length MUP complementary DNAs (cDNAs) corresponding to the five C57BL/6J MUPs and the four BALB/cByJ MUPs excreted in urine MUP7 (EU882230), MUP10/10* (EU882231), MUP12 (BK006654), MUP19 (EU882232), MUP20 (EU882234), MUP3 (EU882235) (Logan et al., 2008) were cloned from male C57BL/6J and BALB/cByJ liver cDNA libraries. MUP4 (EU882229), excreted by neither strain, was cloned from C57BL/6J male submaxilliary gland (Logan et al., 2008). MUPs were subcloned into pMAL bacterial expression vector pMAL-c2X and recombinant MUP protein was produced using the pMAL Protein Fusion and Purification System (New England Biolabs) as previously described (Chamero et al., 2007). rMUPs were presented at 5mg/ml (1X) each for behavioral assays and diluted 1:300 for calcium imaging. Wherever proteins were presented in a blend, each protein was present at 5 mg/ml (for a total of 10-25 mg/ml protein in the blend). For all electrophysiological experiments, rMUPs were washed and exchanged into extracellular/pipette solution ($S_1$, described below) without glucose and maintained in $S_1$ until use.

### Countermarking Behavior

Single 8-week-old BALB/cByJ male mice were housed overnight with a female and then chronically paired with a non-sibling 7-week-old. All tests were carried out under red light. On days 8-9, mice were habituated to the procedure room for 15 min, following which each test mouse was moved to a new clean bedding-free cage lined with Whatman paper to habituate to the test arena. After 5min, the mice were returned to their home cage. On day 10, countermarking response to odor stimuli was assayed by moving mice to a test arena lined with Whatman paper with 50$\mu$l stimulus pipetted onto the center. After 5 min, the mice were returned to their home cage and Whatman paper was collected for quantification. At least 24h was allowed between assays. To visualize urine marks, Whatman paper was sprayed with a 0.3% Ninhydrin solution in Acetone followed by baking at 100°C for 1-2 min, forming a purple complex with urinary proteins. The urine blots were then digitally scanned and the number of spots per sheet was quantified using NIH ImageJ. To discard marks made from footsteps, only marks that were greater than 100 pixels in size were included in the quantification. The stimulus spot, as well as any spots made by fecal matter, were also omitted from quantification.

On the first two days following habituation each mouse was tested with C57BL/6J male urine as a positive control and water as a negative control. These controls were repeated again on two consecutive days at the end of the experiment. Values in all figures represent average number of marks on the first trial of the controls, which were not significantly different from the final controls. For population analysis, total number of marks to each stimulus was scored and only mice that showed at least a 1.5 - fold increase in marking to the positive control as compared to the water were included. All males were screened and only the presumably dominant males, approximately 50% of tested mice, met this criterion. If number of marks to water was 0, then countermarking to test stimulus was considered to be more than half the number of marks deposited in response to the positive control. Some males were tested twice with the same experimental stimulus, however positive and negative controls were also repeated in these animals.

Males raised with rMUP4 exposure were pair housed from 3 weeks of age. A cotton ball with 100 $\mu$l of 5 mg/ml rMUP4 was left in the cage and it was replaced with a new, similarly treated cotton ball at least 8 hr later, for a total of two rMUP4 treated cotton balls per day. A control group was set up who received a clean stimulus-free cotton ball twice per day. At 8 weeks of age, males were split and paired with females overnight, following which they were paired with non-sibling 7- week old males. rMUP4 exposure was continued through formation of dominance hierarchy and marking experiments. To determine the effect of odor experience on adult animals, males were paired housed at 7 weeks old for one week and then assayed for countermarking against C57BL/6J male urine, BALB/cByJ male urine and water on consecutive days. The pairs were then transferred and housed in soiled cages that had previously housed 4 adult C57BL/6J males. The transfer into used C57BL/6J cages took place twice daily to provide continuous C57BL/6J

odor exposure. Weekly, animals were habituated to the testing arena for two days and then the countermarking behavior of dominant males was tested. If the number of marks to BALB/c urine was greater than 1.5 fold of the number of marks to water then the paired mice were switched to clean cages and habituated and tested weekly for countermarking. For Figure 6, BALB/cByJ males were tested with each of the four stimuli at random on four consecutive days. Recorded videos were analyzed using EthoVision to obtain values presented.

## Chemicals, Solutions, and Stimulus Presentation for Electrophysiology

The following solutions were used: a) Extracellular/pipette solution ($S_1$) containing (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES; pH = 7.3 (adjusted with NaOH); osmolarity = 300 mOsm (adjusted with glucose). b) Oxygenated extracellular solution ($S_2$) containing (in mM): 120 NaCl, 25 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 5 BES; pH = 7.3; osmolarity = 300 mOsm. c) Elevated potassium solution ($S_3$) containing (in mM): 100 NaCl, 50 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES; pH = 7.3 (NaOH); osmolarity = 300 mOsm. rMUPs and rMBP were presented at 50 μg/ml each. Stimuli were applied for 3 s (proteins) and 0.5-1 s ($S_3$), respectively, via a software-controlled pressure-driven valve bank connected to an 8-in-1 multi-barrel 'perfusion pencil (Hagendorf et al., 2009; Veitinger et al., 2011).

## Electrophysiology

Acute coronal VNO tissue slices were prepared as described (Hagendorf et al., 2009; Veitinger et al., 2011). VNO slices were transferred to a recording chamber (Slice Mini Chamber, Luigs & Neumann, Ratingen, Germany) on upright fixed-stage video-microscopes (DM LFSA or DM6000 CFS, Leica Microsystems) equipped with apochromatic UVI water immersion objectives for infrared-optimized DIC. Slices were anchored via stainless steel wires with 0.1 mm thick lycra threads and continuously superfused with oxygenated $S_2$ (~3 ml/min; gravity flow; RT). Patch pipettes (5–7 MΩ) were pulled from borosilicate glass capillaries (1.50 mm OD/1.05 mm ID; Science Products, Hofheim, Germany) on a PC-10 micropipette puller (Narishige Instruments, Tokyo, Japan), fire-polished (MF-830 Microforge; Narishige Instruments) and filled with $S_1$. An agar bridge (150 mM KCl) connected reference electrode and bath solution. An EPC-10 amplifier controlled by Patchmaster 2.65 software (HEKA Elektronik, Lambrecht/Pfalz, Germany) was used for data acquisition. Signals were low-pass filtered (analog 3- and 4-pole Bessel filters (–3 dB); adjusted to $^1/_3$ of the sampling rate (5 kHz)). Data were analyzed using Igor Pro 6.22 (WaveMetrics, Lake Oswego, OR) software.

To prevent dialysis of intracellular components, action potential-driven capacitive currents were recorded in 'loose-seal' cell-attached configuration (seal resistance 30-150 MΩ) from VSN somata located deep in the sensory epithelium's basal layer close to the basement membrane. To distinguish stimulus-dependent discharge from baseline noise or random fluctuations, spikes were analyzed using Igor Pro functions (SpAcAn, written by Guillaume Dugué and Charly Rousseau) for detection and analysis of spontaneous events by a threshold/waveform detection algorithm. Inter-stimulus intervals were 30-60 s. Neuronal responses (or the lack thereof) were classified according to a $\Delta f \geq 2 \times SD\ f_{(baseline)}$ criterion.

## Calcium Imaging

Transient increase in free $Ca^{2+}$ concentration in dissociated VSNs was determined by ratiometric fura-2 fluorescence (Chamero et al., 2007; Kaur et al., 2013). Experiments were limited to three stimuli to maintain viability of the neurons. Dissociated VSNs were sequentially exposed to all listed stimuli and response profiles were scored. Neural responses were scored if they met all of the following criteria: (a) $\geq 1.5x$ increase in fluorescence ratio (over baseline signal) during stimulus presentation, and (b) $\geq 1.5x$ increase in fluorescence ratio (over baseline signal) response to the positive control stimulus, c) less than 1.5x (over baseline signal) increase in fluorescence ratio outside the stimulus presentation window.

## Aggression Behavior

Assays were carried out as previously described (Chamero et al., 2007). Wild-type males were individually housed for 7-10 days. A castrated adult swabbed with 50 μl of test solution was introduced into the home cage and behavior was digitally recorded for 10 min. A minimum of 24 hr was allowed to elapse between assays. Videos were analyzed and quantified using Observer software (Noldus Technology). Total duration of aggression was defined as the sum of all aggressive contact behavior consisting of kicking, biting, wrestling or tumbling. Each mouse was tested with a castrate bearing a positive control (whole urine) and a negative control (no stimulus) on two consecutive days prior to the experiment. These controls were repeated again on two consecutive days at the end of the experiment. Only mice that displayed an increase in aggression to a urine swabbed castrate compared to the blank castrate were included in the analysis. Each animal was tested 2-3 times with any given stimulus. All values presented in figures represent an average of all trials with that stimulus.

## Statistics

Behavioral data was tested for normality within each group and homogeneity of variance across groups. Data passing these tests were compared for statistically significant different responses across groups by utilizing a repeated-measures one-way ANOVA, with a Greenhouse-Geisser correction, followed by Bonferroni multiple comparisons test, with individual variances computed for each comparison. For data that did not meet the required by repeated-measures ANOVA assumptions, statistical significance was determined non-parametrically by using Friedman's test followed by a Dunn's multiple comparison test. For all tests, the null

hypothesis of identical responses across groups was rejected at a *p-value* $\leq$ 0.01. All figures report significance as compared to water, unless otherwise noted. VSN activity that was evoked by a given stimulus as well as the BALB/c rMUP pool, and marking response to each stimulus, was compared by using the Spearman's rank correlation test. Statistical significance was calculated, given a null hypothesis, by using a permutation test. The statistical analysis is performed in GraphPad Prism (http://www.graphpad.com/scientific-software/prism/).

## SUPPLEMENTAL REFERENCES

Flanagan, K.A., Webb, W., and Stowers, L. (2011). Analysis of male pheromones that accelerate female reproductive organ development. PLoS One *6*, e16660.

Leypold, B.G., Yu, C.R., Leinders-Zufall, T., Kim, M.M., Zufall, F., and Axel, R. (2002). Altered sexual and social behaviors in trp2 mutant mice. Proc. Natl. Acad. Sci. USA *99*, 6376–6381.

Veitinger, S., Veitinger, T., Cainarca, S., Fluegge, D., Engelhardt, C.H., Lohmer, S., Hatt, H., Corazza, S., Spehr, J., Neuhaus, E.M., and Spehr, M. (2011). Purinergic signalling mobilizes mitochondrial $Ca^{2+}$ in mouse Sertoli cells. J. Physiol. *589*, 5033–5055.
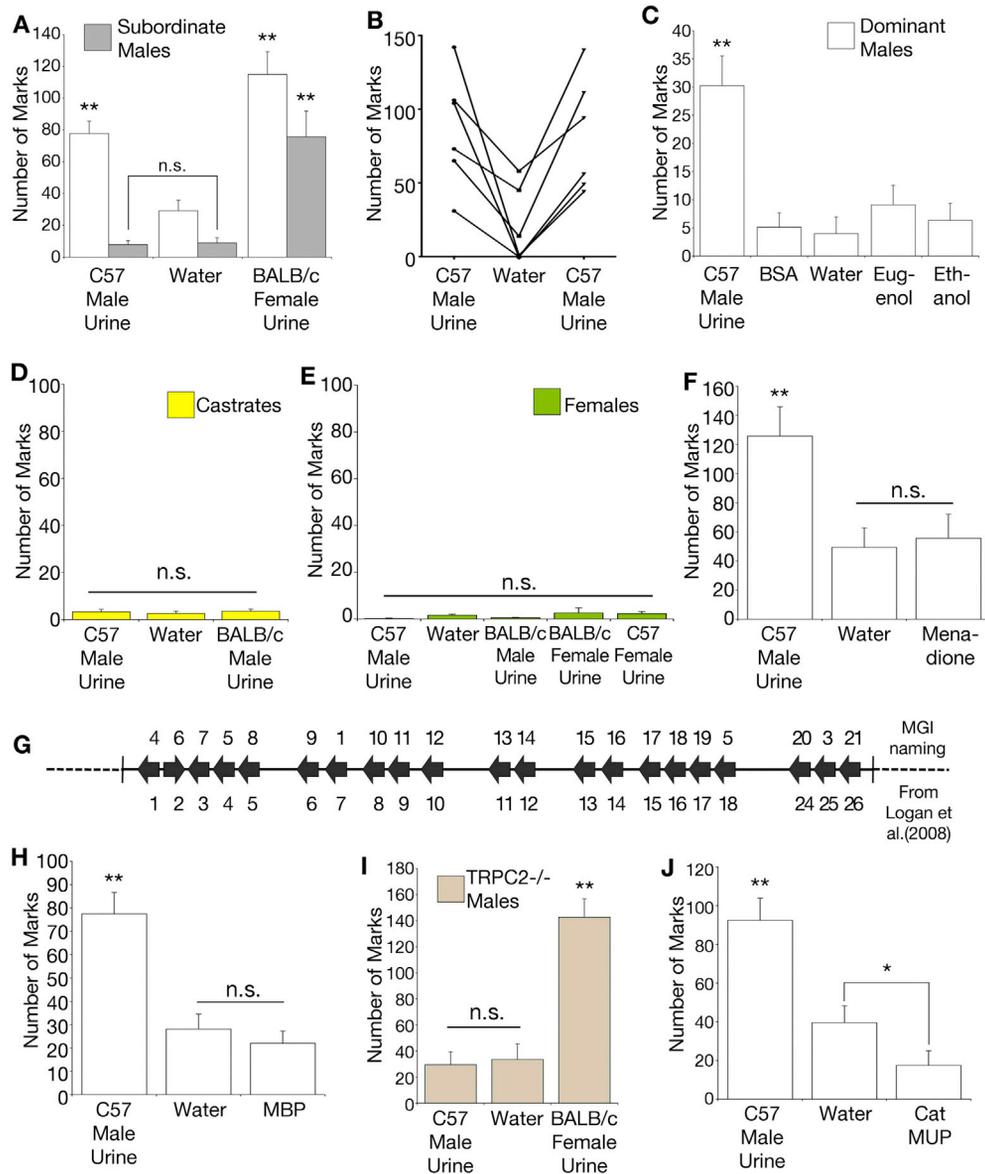
**Figure S1. MUPs Are Sufficient to Elicit Marking Behavior, Related to Figure 1**

(A) Behavioral response of dominant and subordinate males to non-self male and female urine.

(B) Before-and-after plot quantifying the behavior of individual mice over three consecutive days showing consistency of response to controls.

(C) Behavioral response to biologically irrelevant stimuli.

(D and E) Behavioral response of surgically castrated BALB/cByJ males (D) and females (E) to male and female urine.

(F) Behavioral response to menadione, which is used to competitively displace small molecule ligands from MUPs.

(G) *Mup* reference genome annotated with the alternate nomenclature system published by (Logan et al., 2008).

(H) Behavioral response to maltose binding protein, which is used as a purification tag for the rMUPs.

(I) Behavioral response of TRPC2$^{-/-}$ males. This data contradicts previous data showing that TRPC2$^{-/-}$ males display dominant patterns of countermarking (Leypold et al., 2002). Our experiments differ in a number of aspects from the previous analysis including strain background (our TRPC2$^{-/-}$ males are fully backcrossed in the BALB/c background (Flanagan et al., 2011), Leypold et al. used a C57/129 mix), assay set-up (Leypold et al. utilized a live mouse separated from the test animal by a wire mesh, the present assay is entirely olfactory based), or habituation (the present analysis followed two days of habituation, Leypold et al. used animals that were not habituated).

(J) Behavioral response to recombinant cat MUP FelD4, which has been shown to promote fear behavior in mice. n = 8-20. Mean + SEM *p-value*s determined by a repeated-measures one-way ANOVA, with a Greenhouse-Geisser correction, followed by Bonferroni multiple comparisons test or by Friedman's non-parametric test followed by Dunn's multiple comparison test. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. n.s. = non-significant. *p-value*s determined by comparison to water.
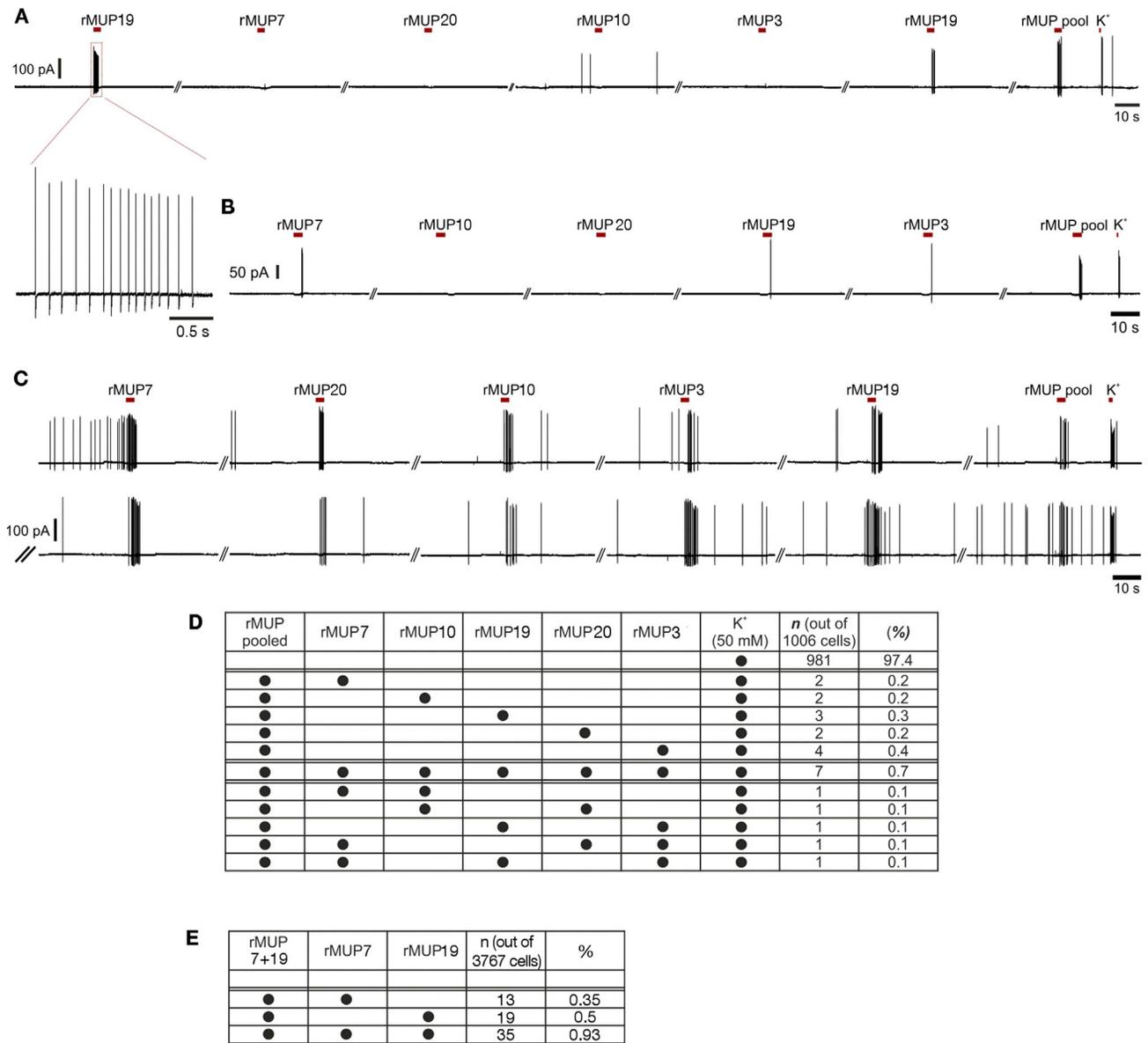
**Figure S2. VSNs Utilize a Combinatorial Code to Detect MUPs, Related to Figure 2**

(A–C) Plot of full-length extracellular neural activity recordings ('loose-seal' patch-clamp) that correspond to the representative traces shown in Figures 2B and 2C. ISI = 60.'' Continuous recordings, interruptions < 1 s (cut marks //). Red bars = stimulus application.

(D) Table showing the different VSN response profiles and their frequency of observation in electrophysiological recordings. 1,006 cells sampled.

(E) Table showing the different VSN response profiles and their frequency of observation in calcium imaging recordings. 3,767 cells sampled.

**D**

| rMUP pooled | rMUP7 | rMUP10 | rMUP19 | rMUP20 | rMUP3 | $K^+$ (50 mM) | $n$ (out of 1006 cells) | (%) |
|---|---|---|---|---|---|---|---|---|
| | | | | | | ● | 981 | 97.4 |
| ● | ● | | | | | ● | 2 | 0.2 |
| ● | | ● | | | | ● | 2 | 0.2 |
| ● | | | ● | | | ● | 3 | 0.3 |
| ● | | | | ● | | ● | 2 | 0.2 |
| ● | | | | | ● | ● | 4 | 0.4 |
| ● | ● | ● | ● | ● | ● | ● | 7 | 0.7 |
| ● | ● | ● | | | | ● | 1 | 0.1 |
| ● | | ● | | ● | | ● | 1 | 0.1 |
| ● | | | ● | | ● | ● | 1 | 0.1 |
| ● | ● | | | ● | ● | ● | 1 | 0.1 |
| ● | ● | | ● | | ● | ● | 1 | 0.1 |

**E**

| rMUP 7+19 | rMUP7 | rMUP19 | n (out of 3767 cells) | % |
|---|---|---|---|---|
| ● | ● | | 13 | 0.35 |
| ● | | ● | 19 | 0.5 |
| ● | ● | ● | 35 | 0.93 |
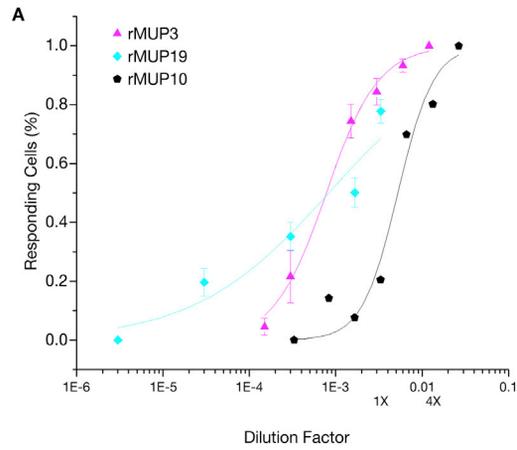
**Figure S3. Combinatorial Code Enables VSNs to Detect Relative Ratios of MUPs, Related to Figure 3**

Concentration dependent response of male VSNs to rMUP3, rMUP19, and rMUP10. Recombinant MUPs (rMUPs) were diluted in PBS, as indicated (x axis) and then assayed by calcium imaging for their ability to stimulate a VSN subpopulation. The dose-efficacy curves reveal a different slope for each ligand. 1X denotes concentration used for most experiments (based on approximate native concentration in urine), 4X corresponds to the concentration used for Figure 3. 1547-4651 cells imaged.

**A**

| Single Self rMUP | Self rMUPs | Self rMUPs +1 | Activated VSNs |
|:---:|:---:|:---:|:---:|
| ● | ● | ● | 15 |
|  | ● | ● | 29 |
|  |  | ● | 24 |

2200 cells imaged

**B**

| Self rMUPs -2 | Self rMUPs -1 | Self rMUPs | Activated VSNs |
|:---:|:---:|:---:|:---:|
| ● | ● |  | 41 |
|  | ● | ● | 12 |
|  |  | ● | 4 |

3893 cells imaged

**C**



**D**



**E**

| rMUP7 | Self - rMUP7 | Self rMUPs | Activated VSNs |
|:---:|:---:|:---:|:---:|
| ● | ● | ● | 66 |
| ● |  | ● | 4 |
|  | ● | ● | 39 |
| 70 | 105 | 109 | 109 |

2555 cells imaged

**F**

| rMUP 10* | Self-rMUP 10* | Self rMUPs | Activated VSNs |
|:---:|:---:|:---:|:---:|
| ● | ● | ● | 22 |
| ● |  | ● | 3 |
|  | ● | ● | 22 |
| 25 | 44 | 47 | 47 |

2873 cells imaged

**G**

| Self rMUPs | Non Self rMUPs | Activated VSNs |
|:---:|:---:|:---:|
| ● | ● | 87 |
|  | ● | 90 |
|  |  |  |
| 87 | 87 | 177 |

2800 cells imaged

**H**

| rMUP12 | Self - rMUP12 | Self rMUPs | Activated VSNs |
|:---:|:---:|:---:|:---:|
| ● | ● | ● | 15 |
| ● |  | ● | 1 |
|  | ● | ● | 54 |
| 16 | 69 | 70 | 70 |

2891 cells imaged

**I**

| rMUP19 | Self - rMUP19 | Self rMUPs | Activated VSNs |
|:---:|:---:|:---:|:---:|
| ● | ● | ● | 24 |
| ● |  | ● | 2 |
|  | ● | ● | 21 |
| 26 | 45 | 47 | 47 |

2115 cells imaged

**J**

| Self rMUPs -2 | Self rMUPs -2 | Self rMUPs | Activated VSNs |
|:---:|:---:|:---:|:---:|
| ● | ● | ● | 42 |
| ● |  | ● | 8 |
|  | ● | ● | 25 |
| 50 | 67 | 75 | 75 |

2809 cells imaged

**Figure S4. The Identity and Ratio of Detected MUP Ensemble Modulate Behavior, Related to Figure 4**

(A and B) Summary quantification of VSN activity, assayed by calcium imaging, as shown in Figures 4C and 4G. Filled circle denotes response to stimulus. Right column = number of VSNs displaying indicated response profile.

(C) Summed behavioral response to self rMUPs −2 (all combinations of two of the four self-rMUPs). n = 76. Mean + SEM $p$-values determined by Friedman's non-parametric test followed by Dunn's multiple comparison test. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. n.s. = non-significant. $p$-values determined by comparison to water.
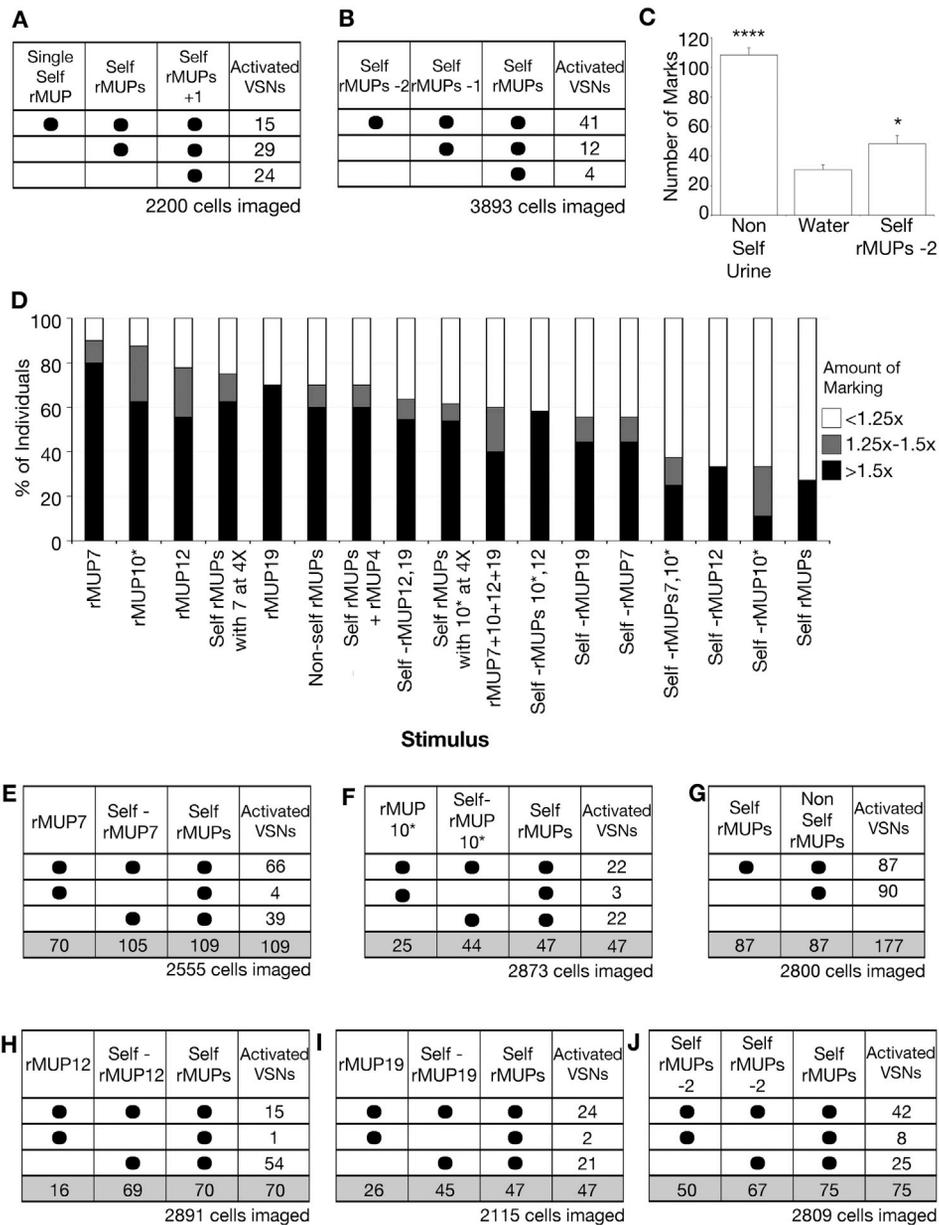
(D) Some rMUP mixtures promoted a reproducible increase in marking whereas other mixtures (like 'self') did not promote marking. Marking behavior of individual mice sorted based on fold increase of number of marks deposited to the stimulus compared to the response to water. Mice were binned according to response > 1.5x, 1.25-1.5x, < 1.25x. Number of mice for each trial per bin (>1.5x, 1.25-1.5x, < 1.25x) are as follows: rMUP7- 8,1,1; rMUP10* −5,2,1; rMUP12-5,2,2; Self rMUPs with 7 at 4x-5,1,2; rMUP19- 7,0,3; non self rMUPs-6,1,3; Self rMUPs+MUP4-6,1,3; Self −rMUP12+19-6,1,4; Self rMUPs with 10* at 4X-7,1,5; MUPs 7+10+12+19- 4,2,4; Self −rMUPs 10*+12-7,0,5; Self − rMUP19-4,1,4, Self − rMUP7-4,1,4; Self − rMUPs 7+10*-2,1,5; Self −rMUP12-3,0,6; Self − rMUP10*-1,2,6; Self rMUPs 3,0,8.

(E–J) Summary quantification of VSN activity, assayed by calcium imaging. VSN activity evoked by rMUP mixtures was directly compared to activity evoked by self-rMUP mixture to determine the percentage of identical neural activity (% VSNs responding self & stimulus, represented in Figure 4I). Filled circle denotes response to stimulus. Right column = number of VSNs displaying indicated response profile. Shaded row shows neurons activated by both stimulus and self.
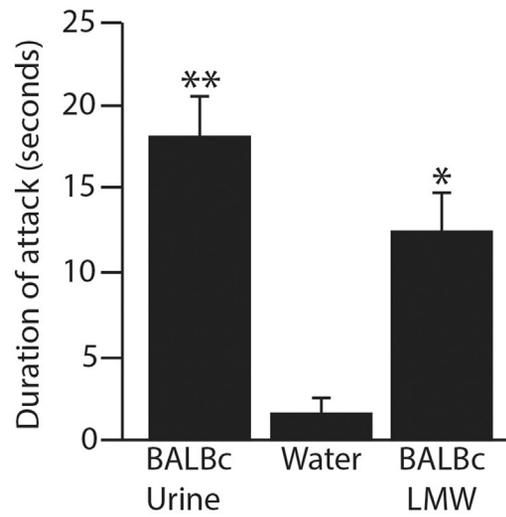
**Figure S5. Signature of "Self" Is Based on Experience, Related to Figure 5**

(A) Quantification of urinary proteins by Bradford assay shows that male mice begin expression of MUPs at approximately 4 weeks of age.

(B) Time required for experience to alter marking behavior. Animals in upper panel (artificial exposure, C57 soiled bedding) correspond to Figure 5E, animals in lower panel (return to self cage) correspond to Figure 5F. Dots = individual animals, lines = mean+/−SEM.

**Figure S6. MUPs Promote Innate Aggression, Related to Figure 7**

Native BALB/c Low Molecular Weight (LMW) fraction promotes aggression in the resident-intruder assay. Black bars = C57BL/6J male residents. n = 5-25. *p-value*s determined by Kruskal-Wallis test **p < 0.01, *p < 0.05. Previously, C57BL/6J urine has been shown to contain two distinct aggression-promoting bioactivities; MUPs and at least one other, currently unidentified, odor cue in the LMW fraction (Chamero et al., 2007). The BALB/c LMW aggression-promoting cue may account for aggressive behavior observed among BALB/c males and to complete BALB/c urine (Figure 7B).