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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