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Message in a bottle: major urinary proteins and their multiple roles in mouse intraspecific chemical communication

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Within a species, sharing of data about identity, sex, health and hormonal status or feeding resources confers an advantage for survival and is actively pursued by linking signalling molecules to specific behavioural or neurohormonal responses. The proper identification of the signals (morphology), their correct use (grammar) and their meaning (semantics) allow us to understand the link between signals and responses. In mice, *Mus musculus*, the identification of meaningful molecules has revealed that both small airborne molecules and custom-tailored proteins are involved in chemical signalling. Among them, the major urinary proteins (MUPs) are barrel-like lipocalins excreted in urine. They bind and transport volatile molecules that may have different meanings, yet MUPs participate in transmitting different pieces of information. Therefore, they are not a simple blend of molecules but a communication system with its own rules to produce, transmit and process information. These actions affect both the physiology and the behaviour of sender and receiver. In fact, functional, behavioural and anatomical specializations allow production and emission of chemosignals by the sender, and proper signal detection and response management by the receiver. Moreover, the response to chemosignals may vary in time, according to internal and external conditions. The mechanism of biological chemical signalling is so efficient and versatile that MUPs are now used as molecular traps to develop biomimetic chemical sensors.

Mice, *Mus musculus*, use a wide array of chemical signals to gain information on the environment and on conspecifics. The emission of chemical signals may modify the behaviour and/or the physiology of other animals, because chemosignals transmit information in the context of communication. Intraspecific communication takes advantage of species-related molecules (pheromones) and individual-specific signature mixtures (see Wyatt, 2010, 2014 for a more comprehensive discussion). We introduce some concepts that evolved in the study of human natural languages, such as morphology, semantics and grammar, to outline a true complex chemical signalling system. Mice excrete signalling molecules in various body fluids (urine, tears, saliva, milk, and possibly other fluids); the first to be studied was urine, since it is collected in relatively large quantity, compared with the other fluids. We outline some ideas that follow from data collected in the last few decades on the role of major urinary proteins (MUPs) in mouse chemosignalling, as they were initially explored in our laboratory, mainly on the CD-1 (Swiss lineage) outbred mouse strain. Other papers in this issue elucidate different major aspects of MUPs chemosignalling, in both laboratory and wild mice.

MORPHOLOGY OF CHEMICAL SIGNALLING: WHICH MOLECULES ACT AS SIGNALS?

Biochemical identification of the structure of signalling molecules has revealed that no common principle underlies the use of a molecule as a signal. This reflects the fact that signals are intrinsically redundant, and numerous chemoreceptors in the oronasal cavities may be activated by different moieties. In the nose, the main olfactory epithelium, the vomeronasal organ, the Grüneberg ganglion and Masera's organ host a variety of chemoreceptors with different functional specificity (Tirindelli et al., 2009). However, the main and accessory olfactory central pathways share connections even at the earliest steps of processing (Mucignat-Caretta, Redaelli, & Caretta, 2012), suggesting a highly integrated signal processing and output definition.

In mice, small, airborne, odorant molecules, as well as peptides and proteins, may be used to communicate. Many small molecules (see below) are synthesized in the body and may be excreted in complex mixtures that act in minute quantities (Redaelli, Orsetti, Zagotto, & Mucignat-Caretta, 2014) to signal some specific mouse

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characteristics at a distance, by attracting/repelling other mice via the stimulation of chemosensory systems. In addition, peptides and proteins also participate in signalling, but these may act in a way different from odorants: as they are coded in the genome, they may give different, more complex and personal information, compared with volatiles. Moreover, they can add their information to that carried by volatiles to create complex mixtures that may ultimately result in different messages.

Most of the identified airborne signalling molecules pertain to different classes and share no chemical properties (Ihara, Yoshikawa, & Touhara, 2013). Different volatiles have been identified mainly from adult male urine, the two most prominent being dehydro-*exo*-brevicomin and 2-*sec*-butyl-4,5-dihydrothiazole (Harvey, Jemiolo, & Novotny, 1989). Being present in all males, their coding capacity allows for sex and status coding, but not individual identity coding (Hurst, 2009). These molecules act as odorants also to the human nose and may act as signalling molecules in invertebrate species, as is the case of dehydro-*exo*-brevicomin, the pheromone of the western pine beetle, *Dendroctonus brevicomis* (Novotny, Harvey, Jemiolo, & Alberts, 1985; Wood et al., 1976). In some instances, signalling through volatile molecules is very detailed; in fact only the (S) stereoisomer of 2-*sec*-butyl-4,5-dihydrothiazole has been identified in male mouse urine and proven to delay countermarking, an opposite result to the (R) stereoisomer of the same molecule (Cavaggioni, Mucignat-Caretta, & Zagotto, 2003).

The most remarkable breakthrough in the identification of mouse chemical cues came with the proof that peptides and proteins participate in chemosignalling. Initially it was postulated that the chemical signals should be volatile to reach the olfactory receptors in the nose, yet in the mouse different biological fluids carry large molecules, including signalling proteins or peptides, directly to the different chemosensory systems within the nose, in particular to the vomeronasal organ (Wysocki, Wellington, & Beauchamp, 1980; see also Figure 1B in Mucignat-Caretta, 2010). In addition to volatile organic compounds, at least four different classes of peptides or proteins may act as chemosignals in mice. As in the case of odorant molecules, they share no apparent characteristics, having different sequences, sizes and shapes.

(1) The lacrimal gland produces and secretes a 7 kDa sex-specific exocrine gland-secreting peptide (ESP). There are 38 ESP members in the mouse species, some of them being expressed in a sexually dimorphic and strain-specific way (Kimoto et al., 2007). One male-specific peptide, ESP1, activates the female vomeronasal neurons by direct contact (Kimoto, Haga, Sato, & Touhara, 2005) through interaction with the vomeronasal receptor V2Rp5 and subsequent stimulation of the amygdala and hypothalamus to increase female sexual receptive behaviour (Abe & Touhara, 2014; Haga et al., 2010).

(2) The androgen-binding proteins (ABPs) are heterodimers linked by disulphide bridges secreted in mouse saliva and then transferred to the fur. They are encoded by a rapidly evolving gene cluster (Karn & Laukaitis, 2009, 2012; Vandewege, Phillips, Wickliffe, & Hoffmann, 2013) and allow the recognition and segregation of mouse subspecies (Bimova, Karn, & Pialek, 2005; Laukaitis, Critser, & Karn, 1997), by promoting assortative mating (Emes et al., 2004).

(3) Signalling peptides and proteins are also excreted in urine: they belong to two main families. First, the major histocompatibility complex (MHC) type I-related peptides have been suggested to participate in chemical communication (Kelliher, Spehr, Li, Zufall, & Leinders-Zufall, 2006; Singh, Brown, & Roser, 1987; Sturm et al., 2013). MHC can, in addition, influence the pattern of odour that characterizes each mouse, making it distinguishable from other mice (Kwak, Willse, Preti, Yamazaki, & Beauchamp, 2010).

(4) Lastly, urine also contains MUPs, small monomeric proteins around 18–20 kDa molecular mass, synthesized by the liver and excreted in the urine (Finlayson, Asofsky, Potter, & Runner, 1965). MUPs have an amino acid sequence homologous to the odorant binding proteins (OBPs) and belong to the same superfamily of proteins, the lipocalins (Böcskei et al., 1992; Cavaggioni, Sorbi, Keen, Pappin, & Findlay, 1987). Despite a low sequence homology, lipocalins share a similar three-dimensional structure, which consists of a barrel made of eight beta-sheets in antiparallel layout, connected by loops and alpha-helices (Lücke et al., 1999). The barrel is closed at one end and surrounds a narrow hydrophobic cavity, lined by highly apolar residues, which may allocate a ligand. MUPs in their native conformation bind odorants with an average affinity around $10^{-4}/10^{-5}$ M for the different ligands (Cavaggioni, Findlay, & Tirindelli, 1990). When excreted in urine, MUPs bind molecules that act as low molecular weight pheromones in mice, mainly 2-*sec*-butyl-4,5-dihydrothiazole and 3,4-dehydro-*exo*-brevicomin, with a hyperbolic binding isotherm indicative of specific binding (Bacchini, Gaetani, & Cavaggioni, 1992; Böcskei et al., 1992). In vitro, the binding reaches equilibrium in 48 h. This, in addition to the unusual thermal stability and insensitivity to cleavage of MUPs, explains why MUPs may retain their binding properties under the most common environmental conditions. The ligands are released by MUPs with different diffusion kinetics at the air/liquid interface, giving a distinctive character to the odorant mark as time goes by. MUPs can also bind xenobiotics, possibly assisting the defence mechanisms inside the body (Kwak et al., 2011; Larsen, Bergman, & Klasson-Wehler, 1990).

In mice, the MUPs family is highly expanded, suggesting a selective pressure for its conservation. Voiding a huge amount of proteins (in the order of several mg/ml) should be a waste of nitrogen and energy (see below); however, the production and excretion of MUPs are advantageous for the individual and the species. The MUPs family evolved in *M. m. domesticus* to support elaborate communication that is relevant for sustaining increased social complexity (Mudge et al., 2008). It comprises 21 fully annotated genes allocated in the MUP locus of mice chromosome 4 (Logan, Marton, & Stowers, 2008), initially described as arranged in tandem pairs, comprising an active gene and a pseudogene (Al-Shawi, Ghazal, Clark, & Bishop, 1989; Clark, Ghazal, Bingham, Barrett, & Bishop, 1985). An interesting insight into the evolutionary history of MUPs showed that central loci could transmit self/nonself information or individuality coding, while peripheral loci could each convey separate specialized functions (Mudge et al., 2008). MUP genes are expressed mainly in the liver (see Fig. 1), from where MUPs are released in the bloodstream and reach the kidneys to be excreted (Clissold, Hainey, & Bishop, 1984). However, MUPs are expressed also in exocrine glands such as lacrimal, salivary and mammary glands (Kuhn, Woodworth-Gutai, Gross, & Held, 1984; Shahan, Denaro, Gilmartin, Shi, & Derman, 1987; Shaw, Held, & Hastie, 1983; Shi, Rodriguez, Shahan, & Derman, 1989), and also in sperm (Zhao et al., 2009) and nasal glands (Utsumi et al., 1999). The potential role as chemosignals of nonurinary MUPs, secreted in different biological fluids, has not been investigated.

Some of the MUP genes are not transcribed: wild mice may express up to 14 MUPs, whereas laboratory strains show a poorer phenotype, which makes them a simplified model to study chemical communication. Besides producing a considerably lesser panel of MUPs than wild mice, there are also significant interstrain differences between laboratory mice, which should warn researchers not to generalize the data to the whole mouse genus or even between strains (Cheetham, Smith, Armstrong, Beynon, & Hurst, 2009).

In summary, chemical signals in the mouse comprise peptides, proteins and small molecules; the latter may act singly or in combination, thus enhancing the coding capacity of the system (Fig. 2).