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Mechanisms of chemical communication: the role of Major Urinary Proteins

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A b s t r a c t . Social communication in the house mouse relies heavily on pheromone-carrying major urinary proteins (MUPs), which delay release of pheromones thus extending longevity of the scent signal. Moreover, MUPs appear to play an important role in individual recognition. In the last few years, new research has led to some important advances. It has been shown that MUPs without their volatile ligands are able to activate neurons in vomeronasal organ and elicit behavioural and physiological responses in the signal receiver. Furthermore, increasing evidence has been found showing that, contrary to the traditional view, MUP expression is condition and state dependent, and that this variation may provide additional information about an individual. Progress has also been made in the description of MUP-like proteins in other rodents; as yet, however, the protein variability typical of the house mouse has not been observed in any other species. Despite these new results, the concept of MUPs has remained more or less unchanged from the date they were first recognized as an identity signal. The aim of this review is to summarise recent knowledge about MUPs and to discuss previous findings in the light of novel facts. Special attention is paid to the consequences the new results may have on our understanding of the individual recognition role of MUPs.

Key words: lipocalins, MUP, *Mus*, mouse, social modulation

Introduction

The polymorphic Major Urinary Proteins (MUPs) belonging to the lipocalin protein family are well known for their role in chemical communication of the house mouse (*Mus musculus*, F l o w e r 1996, C a v a g g i o n i & M u c i g n a t - C a r e t t a 2000, B e y n o n & H u r s t 2003). These relatively small (around 19 kDa) proteins bind a wide range of pheromonally-active ligands that trigger various behavioural and physiological responses in conspecifics (F i n l a y s o n et al. 1963, J e m i o l o et al. 1985, 1986, C a v a g g i o n i et al. 1987, B a c c h i n i et al. 1992, R o b e r t s o n et al. 1993, N o v o t n y et al. 1999a). Among the multiple roles that have been ascribed to MUPs, a fundamental one is the modification of ligand release dynamics. MUPs decrease the evaporation rate of pheromones from deposited scent mark by retaining these volatile compounds in the binding cavity. Though this may lead to a slightly reduced intensity of the scent signal, the overall effect is beneficial because it considerably prolongs the period for which the signal is detectable thereby reducing the number of times scent marks need to be refreshed (H u r s t et al. 1998, B e y n o n et al. 1999). Moreover, MUPs facilitate the effective transportation of hydrophobic ligands in body fluids, and concentrate them in secretions including not only urine, but also saliva and tears (Shaw et al. 1983, B a c c h i n i et al. 1992).

MUPs are expressed in several mouse tissues including liver, salivary, lachrymal and mammary glands from which they are then transported to the appropriate secretion

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site (Finlayson et al. 1965, Shaw et al. 1983, Shahan & Derman 1984, Shahan et al. 1987). From the liver MUPs are transported by blood to the kidney and excreted in the urine (Rümke & Thung 1964). Expression is regulated by different mechanisms which lead to quantitatively and qualitatively specific MUP phenotypes in each tissue (Hastie et al. 1979, Shaw et al. 1983). Tissue-specific differences may be caused by polymorphism in the promotor or regulatory binding-site (Krauter et al. 1982). Particular role may also play heritable epigenetic modifications, the mechanism of which is not well understood yet (Sanford et al. 1987, Roemer et al. 1997, Mudge et al. 2008). The most important factors regulating MUP expression appear to be the age, sex, species and hormonal status of the individual (Wicks 1941, Derman 1981, Knopf et al. 1983, Sampsell & Held 1985, Johnson et al. 1995, Robertson et al. 2007). Recently, it has been reported that the social environment is other factor that modulates MUP expression (Stopka et al. 2007).

MUPs are coded by multiple genes clustered on chromosome 4 (Bishop et al. 1982, Krauter et al. 1982). The genes can be divided into two groups. An older group is more divergent and incorporates genes and pseudogenes. The second group shows high sequence similarity, indicating its origin from the single pair of gene and pseudogene. Pseudogenes contain stop codons and mutations in their sequences which preclude translation into the protein capable of semiochemical binding (Clark et al. 1985, Logan et al. 2008). However, it has been proposed that pseudogenes could be sometimes translated and produce hexapeptide that is thought to possess some biological activity (Vandenbergh et al. 1976, Mucignat-Caretta 1995, More 2006). So far, 21 different *Mup* genes and similar number of pseudogenes have been identified (Logan et al. 2008, Stopkova et al. 2009, in this issue).

The expression of different alleles of multiple MUP loci gives rise to the unique phenotypic pattern consisting of up to fifteen distinct MUP isoforms in each individual (Robertson et al. 1997, Pes et al. 1999, Payne et al. 2001). This MUP pattern differentiates an individual from other members of the species, but simultaneously reflects genetic similarity. It has been demonstrated that mice *Mus musculus domesticus* are able to use differences in MUPs to recognise the identity of conspecifics (Hurst et al. 2001a, Cheetham et al. 2007). Though the evidence for the individual recognition role of MUPs has gained quite strong support, there are some unresolved questions that deserve further discussion. The stability of MUP expression through time, which has been emphasized several times as an important presumption for identity signalling but which had not been sufficiently tested till recently (Hurst et al. 2001b, Nevison et al. 2003), has not been confirmed in some recent studies indicating the need for more thorough investigation (Stopka et al. 2007).

New findings have also corroborated the lingering speculation that MUPs themselves possess information that may elicit behavioural or physiological response in the receiver, in the same way as do their pheromonal ligands (More 2006, Chamero et al. 2007). Two mechanisms of transmission of the signal carried by MUPs had been proposed. Today, the direct model – according to which MUPs directly associate with olfactory or vomeronasal receptors – is the favoured alternative (Sharrow et al. 2002).

The alternative possibility - that the MUP signal is reflected in bound ligands which subsequently activate the appropriate receptor - should not be neglected. This indirect model is based on the assumption that MUP isoforms bind pheromones with different binding affinities and release them with different speed thus forming a specific evaporation profile

corresponding to a particular MUP pattern. This implies that the variation among isoforms is concentrated in the amino acid sequence of the region that forms the binding cavity of the protein (Marie et al. 2001, Veggerby et al. 2001). Though few studies have identified such amino acid changes, the majority of isoforms exhibit sequence variation on the surface of molecule where it cannot have any effect on binding properties. Therefore, the applicability of the indirect model is, in this context, limited (Sharrow et al. 2002).

In the last few years focus has also been aimed at identifying the pheromone-carrier proteins in other rodent species. Though MUP orthologues have been identified in several species more or less related to *Mus musculus*, first results indicate that in detail the role of these proteins may differ between species (Robertson et al. 2007, Beynon et al. 2008).

In the following section we will summarise different factors that have been shown to affect MUP expression, and discuss the relevance of particular studies and their contribution to the understanding of MUP role in communication of the house mouse. The second section will be dedicated to the direct signalling role of MUPs via the vomeronasal organ and the effects that they trigger in the signal receiver. In the third section the impact of new results on our understanding of individual recognition will be discussed. Finally, the information about the current knowledge of the presence of MUP similar proteins in other rodent species will be provided. Since urine is the most important source of chemical signals in the house mouse we will focus on the urinary MUPs, which is where the majority of research has been to date.

Factors modulating MUP expression

Hormones

Studies using testosterone treated mice demonstrated high dependence of MUP gene activity on testosterone level (Wicks 1941, Thung 1956). When treated with testosterone, castrated males, females and juveniles all showed MUP expression levels typical for intact adult male (Rümke & Thung 1964, Hastie 1979). The induction was both quantitative and qualitative, meaning that the complexity of MUP pattern in testosterone treated females was the same as in adult males. These results demonstrate selective dependence of different MUP genes on testosterone regulation as some of them are transcribed quite actively in absence of testosterone, while others are induced only after the testosterone administration (Clissold et al. 1984).

Other hormones involved in the regulation of liver MUPs are growth hormone and thyroxine. Knopf et al. (1983) demonstrated that mice deficient in growth hormone or thyroxine exhibit low levels of MUPs in the urine. Administration of the missing hormone partially or totally restored the quantity and complexity of MUPs. Moreover, simultaneous administration of both hormones to hypophysectomised females resulted in the over expression of MUPs to a level three fold higher than in normal males (Johnson et al. 1995).

Several studies have reported that distinct urinary MUPs are regulated differentially. Whereas some MUPs are increased by administration of each of the three hormones, others are induced by only in response to a particular hormone (Knopf et al. 1983, Kuhn et al. 1984). The significance of this differential regulation of expression is unknown. However, the present results indicate that the mechanism of regulation underlies the role specificity of different MUP isoforms.

Although these studies have presented important information about the hormonal regulation of MUPs, their relevance is limited. They suffer from several shortcomings which substantially complicate interpretation of the biological meaning of the results. First, the animals had been usually treated with unnaturally high hormone levels, or in the case of females with hormones that are normally virtually absent. Second, a specific regime of hormone administration which reflects natural fluctuation in its levels may be required to identify the precise effect. Finally, the relevance of these results for wild animals is questionable because animals used in such studies are usually severely affected by the experimental treatment in such way that they would probably not survive in the natural environment. We have scarce information about MUP hormonal regulation in the natural environment where gradual and more subtle changes rather than total depletion of one or a few hormones occur. In other words, the research focused on intact animals which undergo common physiological changes is now needed to assess the biological importance of different regulating factors.

Fortunately, in the past few years this topic has started to gain some research attention. Stopka et al. (2007) characterized the quantitative variation of MUP expression during the estrous cycle in C57Bl/6 mice. They found that the expression level of urinary MUPs fluctuated over the course of the estrous cycle, reaching its maximum at the beginning of estrus. This study suggests that, in females, MUPs are under the control of hormones regulating reproductive physiology and may be used to advertise forthcoming period of fertility of the female in order to attract potential mates.

Age

Liver MUP mRNA is undetectable in juvenile males until the age of approximately 21 days, after which age it steadily rises reaching the normal adult level at about two month (Derman 1981, Barth et al. 1982, Rusu et al. 2008). Likewise, the complexity of MUP patterns - represented by the number of MUP isoforms expressed - has been reported to be lower in juvenile than in adult mice (Payne et al. 2001). However, this may not be a common rule. Payne et al. (2001) reported that 20 days old females express the same quantity of protein as do adults of this sex indicating different mechanisms of developmental regulation in males and females. Moreover, Rusu et al. (2008) observed that complexity of MUPs had not differed at 21 and 61 days of age in pairs of brothers exhibiting early onset of agonism. In males that stayed amicable until the age of two months the increase of MUP complexity was delayed until this time. Since the expression of MUP is androgen dependent (Wicks 1941, Thung 1956), the simplest explanation is that more complex juvenile MUP patterns are associated with higher levels of testosterone that concurrently cause the earlier onset of aggression (Compaan et al. 1994). However, if this is the case, one would expect the elevation of both complexity and quantity of MUPs (Rümke & Thung 1964, Hastie 1979), which was not observed in the study. Therefore, it seems that regulators which influence the complexity of MUP patterns in juveniles are not limited to testosterone and that the developmental regulation of MUP quantity and complexity is mediated by different regulatory agents.

Rusu et al. (2008) revealed that two particular bands were present in the MUP patterns of juveniles that became aggressive sooner. This may indicate that there is a functional specificity of the roles of different MUPs, and particularly that these two MUP isoforms are associated with aggressive behaviour in males.

It is known that exposure to the MUPs of other males promotes aggression in adult male mice and stimulates competitive countermarking (Humphries et al. 1999,

Chamero et al. 2007, see section 2). Thus, it is surprising that a difference in the presence of two aggression-promoting MUP isoforms was not observed in adults where neither the concentration nor the complexity was correlated with the level of agonism (Rusu et al. 2008). A possible explanation for this finding is that the more complex MUP pattern of juveniles, caused by the expression of the aggression-promoting isoforms, may result in higher level of aggression directed to these individuals by adult males. In coevals, the exposure to these isoforms may induce earlier onset of agonism. Nevertheless, it remains unclear if the expression of particular MUP isoforms has direct association with aggression. To address this question, experiments in which the level of aggression and complexity of MUP pattern are directly examined in adult as well as juvenile males are required.

Sex

The level of sexual dimorphism in MUP expression, with males producing considerably more MUPs than females, was reported for the first time 68 years ago (Wicks 1941). Partly for higher MUP production, the major part of research has been carried out on males. In comparison we know little about MUP roles and expression regulation in females. However, their significance in females should not be underestimated simply on the basis of a lower expression level in this sex (Stopka et al. 2007).

Important information which may help us to understand both the interspecific and intraspecific roles of MUPs is the level of sexual dimorphism. Substantial variation in this parameter among different species and even subspecies of wild mice (genus *Mus*) has been reported. The highest sexual dimorphism at the level of mRNA has been observed in *M. spretus*, where males produce a hundred times more liver MUP mRNA than do females (Sampsell & Held 1985).

In the two subspecies of the house mouse *M. m. musculus* and *M. m. domesticus*, MUP quantity has been analysed at the mRNA and protein expression levels, and a specific degree of sexual dimorphism has been found in each subspecies. In *M. m. musculus,* differences between males and females are much more pronounced than in *M. m. domesticus* subspecies, which is particularly interesting in the context of known hybridization between "*musculus*" and "*domesticus*" in the European contact zone spanning from Denmark to Bulgaria (Boursot et al. 1993, Stopkova et al. 2007). Higher production of MUPs in *M. m. musculus* males over *M. m. domesticus* males may be responsible for the different mating preferences of the females of the two subspecies that has been demonstrated in several studies. *"Musculus*" females prefer mating with their own subspecies whilst "*domesticus*" females do not display consistent preference even when they are given a choice between self or other *Mus* species (Munclinger & Frynta 1997, Christophe & Baudoin 1998, Smadja & Ganem 2002, Smadja & Ganem 2004, Bímová et al. 2005). These results indicate that MUPs may be an important part of a subspecies – and hence perhaps species – recognition system that maintains homospecific mating, at least in *M. m. musculus*, and that the process of mate choice may vary in detail between *Mus* species or subspecies. Whilst the smaller "*musculus*" may rely heavily on the MUP quantity when advertising its qualities to females, the superior competitive ability of the more aggressive and larger "*domesticus"* may be sufficient to ensure access to reproduction (Thuesen 1977, van Zegeren & van Oortmerssen 1981, Stopkova et al. 2007). However, it may be possible that MUP quantity is an important factor influencing mate choice in both subspecies. The lack of homo(sub)specific preferences in *M. m. domesticus* females observed in several studies may be then explained by the

conflict in decision making between the male with higher MUP quantity and the male of the same subspecies.

Signalling role of MUPs

Pheromonal ligands of MUPs trigger various behavioural and physiological effects that mediate complex social relationships. Among the many effects that pheromones trigger in mice, the modulation of female reproductive physiology by both male and female chemical signals is perhaps the best described (e.g. Jemiolo et al. 1986, Ma et al. 1999, Novotny et al. 1999b). A detailed description of pheromonal activity exceeds the scope of this review, however Table 1 summarises some of the more widely discussed roles in order to illustrate the diversity and importance of pheromonally mediated processes in mice.

MUPs had been largely considered to be pheromonally inactive because of their high molecular weight, which limits their volatility and thus their availability to the recipient (Novotny et al. 1999a). However, it has been proposed that MUPs may be transferred to the sensory tissue of the vomeronasal or main olfactory organ by direct contact of the nose of recipient with the scent mark. Support for this hypothesis has been provided by several studies that have shown that mice respond differently to urinary scent mark when allowed full contact compared to when contact is prevented (Humphries et al. 1999, Moncho-Bogani et al. 2002, Nevison et al. 2003, Martinez-Ricos et al. 2007). An indication of the potency of MUPs is given by the observation that these proteins are well known human allergens (Hollander et al. 1999, Renström et al. 1999, Thulin et al. 2002), making it apparent that even microsmatic humans with no functional vomeronasal organ have no difficulty in getting MUPs into their sensory systems.

Despite the recent debates on the transmission process of MUPs, the suggestion that they possess the pheromonal activity themselves and influence the reproductive physiology of females was originally raised in 1975 (Vandenbergh et al. 1975). Later, Mucignat-Caretta et al. (1995) reported that MUP, as well as N-terminal hexapeptide derived from MUP pseudogenes, accelerates sexual maturation in female mice. Though this finding had not been confirmed (Novotny et al. 1999a), the question of whether MUPs themselves possess pheromonal activity has attracted attention of researchers from that time onward.

Recently, support for the signalling role of MUPs without the participation of their ligands was provided by Chamero et al. (2007), who reported that MUPs isolated from male mouse urine as well as recombinant MUPs (rMUPs) prepared in *E. coli* activated a subset of receptors (V2R) in the vomeronasal organ. The behavioural assay carried out along with the analysis of vomeronasal neurons' responses revealed that both MUPs and rMUPs applied on the fur of castrated males promoted aggression in other males.

It is not known if the aggressive response was triggered by all MUPs or by a specific subset of isoforms. However, several findings support the latter possibility. The role of specific MUP isoforms in the timing of onset of aggressive behaviour in males (Rusu et al. 2008) and differential dependence on testosterone has already been mentioned. Moreover, it is known that MUPs are produced in both sexes with some overlap between the MUP patterns (Robertson et al. 1997, Pes et al. 1999, Payne et al. 2001, Armstrong et al. 2005). If all MUPs elicit aggressive behaviour, females would be attacked equally as frequently as males, which is apparently not optimal strategy and disagree with the observations of mouse behaviour.

Table 1. Pheromonally mediated behaviours in the house mouse (*Mus musculus*).

Effect	Compounds	Source	References
Females			
Puberty acceleration (Vandenbergh effect)	α- and β-farnesenes	Male preputial gland	Vandenbergh 1969 Vandenbergh et al. 1975
	6-hydroxy-6-methyl-3-heptanone	Male urine	Novotny et al. 1999a
	2-*sec*-butyl-4,5-dihydrothiazole	Male urine	
	2,3-dehydro-*exo*-brevicomin	Male urine	
Puberty delay	2,5-dimethylpyrazine	Female urine	Cowley & Wise 1972 Vandenbergh et al. 1972 McIntosh & Drickamer 1977 Novotny et al. 1986 Jemiolo & Novotny 1994
Oestrus synchronization (Whitten effect) and extension	2-*sec*-butyl-4,5-dihydrothiazole	Male urine	Whitten 1956 Whitten 1958
	2,3-dehydro-*exo*-brevicomin	Male urine	Bronson & Whitten 1968 Jemiolo et al. 1986
	α- and β-farnesenes	Male preputial gland	Jemiolo et al. 1989 Ma et al. 1999
	2-heptanone	Male preputial gland	
Pregnancy block (Bruce effect)	Low molecular constituents	Male urine	Bruce 1959 Peele et al. 2003
Attractant	2-*sec*-butyl-4,5-dihydrothiazole	Male urine	Jemiolo et al. 1985 Jemiolo et al. 1991 Lin et al. 2005
	2,3-dehydro-*exo*-brevicomin	Male urine	
	α- and β-farnesenes	Male preputial gland	
	(methylthio)methanethiol	Male urine	
Males			
Impaired spermiogenesis	2,5-dimethylpyrazine	Female urine	Daev & Dukelskaya 2003
Stimulation of aggression	2-*sec*-butyl-4,5-dihydrothiazole	Male urine	Novotny et al. 1985
	2,3-dehydro-*exo*-brevicomin	Male urine	
Aversive signal	α- and β-farnesenes	Male preputial gland	Novotny et al. 1990 Jemiolo et al. 1992

Beside the pheromonal role of MUPs in mediating inter-male aggressive behaviour, the impact of MUPs on reproductive physiology of females has recently come under investigation. Following on the study of Mucignat-Caretta (1995), purified MUP and the N-terminal hexapeptide of MUP pseudogene have been tested for activity in enhancing ovulation in female mice (More 2006) with positive results. This study raises a number of questions. It is not clear whether the physiological effect of the whole MUP is elicited by its N-terminal sequence, which shares four amino acid residues with the pseudogene product, or by another part of the molecule. Furthermore, though there are some indications of the presence of the N-terminal hexapeptide in mouse urine (Vandenbergh 1976), this has not been demonstrated directly. Thus, though we have now evidence indicating the importance of MUPs as an active pheromone, further research is needed to elucidate the identity of active compound involved in the modulation of female mouse physiology.

Although a pheromonal signal may in principle be released constantly, generally we expect production to be carefully timed to ensure activation of each pheromone in the appropriate situation which can be achieved by action of different internal and external regulating factors. A well known example is the production of specific volatiles in the pheromonal profile of males of different social status (Apps et al. 1988, Novotny et al. 1990) where pheromone regulation is usually attributed to the level of androgens. Interestingly, MUP concentration which is known to be highly dependent on the testosterone, appears to be similar in adult males of different social status (Hurst et al. 2001b). However, testosterone may still play the role in modulation of their production. For instance, a sufficient level of testosterone may be important to enable immediate increase of MUP expression when it is needed, for example when a reproductively active female is encountered.

A further factor that has considerable effects on pheromone levels in mice is the social environment (Bronson 1979, Novotny et al. 1999b). It has recently been demonstrated that this factor is also an important modulator of MUP production. MUP concentration increased in mice that were exposed to the presence of the opposite sex in comparison with isolated individuals (Stopka et al. 2007).

MUPs and individual recognition

An explanation for the biological significance of MUP polymorphisms had been a subject for research in various laboratories for a long time (Marie et al. 2001, Sharrow et al. 2002). The opinion that prevails today has been proposed by Hurst and her colleagues (2001a). They observed an extensive inter-individual heterogeneity of MUP pattern in populations of wild mice of *Mus musculus domesticus* and suggested that mice can exploit this MUP specificity to discriminate between conspecifics (Robertson et al. 1997, Pes et al. 1999, Payne et al. 2001). They also provided evidence for this claim with the finding that male mice are able to distinguish between two scent marks differing only in MUP pattern (Hurst et al. 2001a). Although this did not imply that mice remember the identity of an individual on the basis of its MUP pattern using it subsequently to recognise this individual among others, which is the true definition of individual recognition (Dale et al. 2001, Thom & Hurst 2004, Tibbetts & Dale 2007), it strongly indicated that this may be the case.

Recently, the identity signalling role of MUPs has been confirmed using the known preference of females for males who countermark the scent marks of competitors, and

thereby advertising territory ownership. It has been demonstrated that females reliably recognise the identity of the countermarking male on the basis of his scent mark, which they previously encountered (Cheetham et al. 2007). In this study the response was apparent only when the scent mark of countermarking male has differed from the other male's scent mark in MUP pattern. When there was a genetic difference between males at the major histocompatibility complex (MHC), which has been many times suggested as a candidate identity signal, females failed to respond differently to the countermarking and countermarked males. Thus, in wild derived mice of *Mus musculus domesticus* MUPs rather than MHC are the main determinants mediating individual recognition.

The information which mice could acquire from MUPs is, however, much more complex than simply an identity signature. MUP phenotypes are the product of the individual's genes, which are inherited from both parents and may thus provide information about relatedness to other members of the population. This is particularly important in the context of avoiding inbreeding due to mating with close relatives.

Inbreeding avoidance mediated by MUPs has been tested by Sherborne et al. (2007), who reported that in wild derived mice *M. m. domesticus*, fewer offspring were produced by animals sharing both (but not one) MUP haplotypes. However, in their experimental group only 31 % of full siblings had both MUP haplotypes in common and as the authors observe, the level of haplotype sharing is likely to be even more infrequent in natural populations. Moreover, maturing males are known to disperse while females usually stay in the natal territory (Lidicker 1976, van Zegeren 1980). Considering this data, it must be pointed out that biological significance of MUPs in inbreeding avoidance is controversial because the probability of encountering the male that shares both MUP haplotypes with female is low and relatives that share only one MUP haplotype would not be avoided as mating partners at all.

It is possible that in the populations of wild living mice other mechanisms, for example sex-biased dispersal, provide the primary mechanism for avoiding inbreeding (Lidicker 1976, van Zegeren 1980, Rusu & Krackow 2005). Then, MUPs may be used in a slightly different but related context. The avoidance of the same-MUP pattern individuals result in the production of offspring with higher heterozygosity in the MUP loci, and due to the highly polymorphic nature of MUPs these individuals are also likely to be more heterozygous across the genome. Moreover, it has been recently demonstrated that MUPs may be used for assessment of heterozygosity and that females prefer to associate with MUP heterozygous over MUP homozygous males (Thom et al. 2008). Therefore, in terms of mate choice it would be advantageous for mice to produce offspring with high level of MUP heterozygosity.

Though we now have strong support for the involvement of MUPs in the process of individual recognition in *Mus musculus domesticus*, there remain some confounding points that limit a proper understanding of MUP functioning in this context. One of the most important issues concerns the poorly tested assumption about the stability of MUP expression through time. According to the prevailing view MUPs are considered to be a fixed characteristic of the individual, which is not dependent on metabolic and environmental variation providing a stable identity signature (Hurst et al. 2001b, Nevison et al. 2003). However, the studies mentioned in this review demonstrated that the intrinsic and extrinsic factors play a non-negligible role in influencing MUP expression (More 2006, Stopka et al. 2007). Substantial dependence of MUP expression on several different hormones, and high levels of tissue- specificity also evoke the question of why should this

system, the expression of which is supposed to be stable, be dependent on such sophisticated hormonal regulation (Knopf et al. 1983, Kuhn et al. 1984). To what extent this variation can interfere with the individuality signalling role is currently unknown. If the changes of MUP expression are gradual so that members of the social group have time to adjust to the changing signal, the effect of variation on the identity signature role may be minor (Thom & Hurst 2004).

The necessity of direct contact with the scent mark, reported several times, suggests that MUPs themselves with no bound ligands may mediate individual recognition (Humphries et al. 2001, Nevison et al. 2003). Though the activation of VNO neurons by MUPs has been confirmed in other contexts (Chamero et al. 2007), we have no information how the process of perception of the identity signal proceeds. It is implied that there should be high specificity between particular MUPs and VNO receptors or that the action potential elicited by specific MUP isoforms is different. Thus, the next important step is to identify appropriate receptors and analyse their possible specificity for MUP isoforms. To achieve this detailed description of amino acid sequence of MUP isoforms is necessary. This is, because of the high level of MUP polymorphism (Logan et al. 2008), a far from easy task.

MUP similar proteins in different rodent species

The presence of MUPs or their equivalents has been, reported in the genus *Rattus*, and more recently in other rodents of genus *Mus*, *Phodopus, Myodes* and *Mastomys* (Robertson et al. 2007, Beynon et al. 2008, Daniszova et al. 2009, in this issue) Recent analysis of *Mup* gene cluster revealed that MUP expansion occurred independently in the house mouse and the rat, whose common ancestor shared only one or few *Mup* genes. The number of *Mup* genes and pseudogenes in the rat is nine and thirteen respectively which is approximately half as many as are found in the house mouse (Logan et al. 2008). Surprisingly, investigation of the urine of wild rats *Rattus norvegicus* has not revealed any MUP heterogeneity (Beynon et al. 2008).

This lack of phenotypic heterogeneity at the protein level was also observed in *Mus macedonicus* and *Mus spretus* (Robertson et al. 2007). This indicates that the expansion of *Mup* gene cluster did not emerge immediately after the divergence of the genus *Mus* and *Rattus,* but more recently, after the divergence within the *Mus*. Thus, among the species surveyed so far, extensive MUPs polymorphism is typical only for *Mus musculus*. In fact, phenotypic heterogeneity (at the protein level) has been directly demonstrated only in the "*domesticus"* subspecies (Robertson et al. 1997, Pes et al. 1999, Payne et al. 2001). This suggests that the involvement of MUPs in the identity signalling could be limited to only one species. However, testing this assumption requires analysis of *Mup* content in other *Mus* species, with special attention to the complex *Mus musculus*.

Information about MUPs in other rodent species is very limited. However, it is evident that none of the species tested so far exhibits phenotypic variation of MUPs comparable to that found in the house mouse (Robertson et al. 2007, Beynon et al. 2008). The precise role of the pheromone carrier in the particular species may be influenced by its ecology. Since rodent species in which MUP-like proteins have been discovered so far differ in their ecological requirements it is possible that they use pheromone carriers for different communication purposes. To address this question there is a need to analyse more rodent species, including sequencing their genomes and thorough investigation of putative pheromone-carriers on the protein level.

An interesting conjunction when debating the variable roles of MUPs in different species arises from the known scavenger function of many lipocalin proteins (L e c h n e r et al. 2001, D a v i d et al. 2003, G r o l l i et al. 2006). Moreover, P e t r a k et al. (2007) showed that MUP expression is enhanced in mice afflicted with hereditary hemochromatosis I. Thus, it seems likely that in some species MUPs may be involved in iron transportation. Alternatively, this may be their original function from which they have been co-opted for additional social functions (for more information see S t o p k o v a et al. 2009, in this issue).

Conclusions

With the knowledge we have about major urinary proteins, it is beyond doubt that their role in chemical communication exceeds the sole function of pheromone carrier. However, there still remain many unanswered questions about the precise role of MUPs, even in such well-researched species as the house mouse. In other rodents the exploration of lipocalin pheromone-carriers is merely at its beginning. To broaden our knowledge, a multidisciplinary approach and utilization of different research tools is necessary. The analysis of MUPs and similar proteins should be carried out at the DNA level as well as at the level of mRNA and protein expression. Moreover, a proper understanding of the roles of MUPs requires the integration of several disciplines spanning from neurology and physiology to behavioural ecology and phylogenetics. The research should be primarily focused on free-living animals in which the inter-individual heterogeneity of MUPs is apparent, and whose behavioural and physiological responses to chemical stimuli are not changed by intensive selection in captivity.

Novel studies have provided evidence that MUPs may be employed as a source of fixed, inherited characteristic of individuality, simultaneously reflecting information on immediate status. We suggest that this high informational potential may be facilitated by the heterogeneity of MUPs and the functional specificity of different MUP isoforms.

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