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Roles for learning in mammalian chemosensory responses

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Abstract

A rich variety of chemosignals have been identified that influence mammalian behaviour, including peptides, proteins and volatiles. Many of these elicit innate effects acting either as pheromones within species or allelochemicals between species. However, even innate pheromonal responses in mammals are not as hard-wired as the original definition of the term would suggest. Many, if not most mammalian pheromonal responses are only elicited in certain behavioural or physiological contexts. Furthermore, certain pheromones are themselves rewarding and act as unconditioned stimuli to link non-pheromonal stimuli to the pheromonal response, via associative learning. The medial amygdala, has emerged as a potential site for this convergence by which learned chemosensory input is able to gain control over innately driven output circuits. The medial amygdala is also an important site for associating social chemosensory information that enables recognition of conspecifics and heterospecifics by association of their complex chemosensory signatures both within and across olfactory chemosensory systems. Learning can also influence pheromonal responses more directly to adapt them to changing physiological and behavioural context. Neuromodulators such as noradrenaline and oxytocin can plasticise neural circuits to gate transmission of chemosensory information. More recent evidence points to a role for neurogenesis in this adaptation, both at the peripheral level of the sensory neurons and via the incorporation of new neurons into existing olfactory bulb circuits. The emerging picture is of an integrated and flexible response to chemosignals that adapts them to the environmental and physiological context in which they occur.

Introduction: what are pheromones?

Pheromones were first defined by Karlson and Lüscher over 50 years ago as "substances secreted to the outside of an individual and received by a second individual of the same species in which they release a specific reaction, for example a definite behaviour or developmental process" (Karlson and Lüscher, 1959). First identified in silk moths (Butenandt et al., 1959), many examples have since been identified in insects and have important practical applications in pest control. However, our knowledge and understanding of vertebrate and mammalian pheromones, which are the focus of this review, has lagged appreciably behind that of insects. Indeed some have questioned whether mammalian pheromones really exist (Doty, 2010). However, the ever-growing number of examples of substances that meet the original definition of a pheromone provide convincing evidence that pheromonal effects do occur across a range of mammalian species. However, the evidence for pheromonal effects is less strong in apes and humans in which the importance of visual and verbal modes of communication has led to the evolutionary decline in olfactory capability in general (Kambere and Lane, 2007).

When Karlson and Lüscher first proposed their definition of a pheromone they envisaged that their definition would be redefined and updated over time (Karlson and Lüscher, 1959). Yet it still forms the core of most accepted definitions, such as the recent, slightly modified definition by Wyatt, "molecules that are evolved signals, in defined ratios in the case of multiple component pheromones, which are emitted by an individual and received by a second individual of the same species, in which they cause a specific reaction, for example, a stereotyped behavior or developmental process." (Wyatt, 2014). Others have added their own additional requirements such as that a pheromone must be airborne (Stern and McClintock, 1998). But there are whole classes of involatile substances that have pheromonal effects following direct physical contact, so this is definitely not a requirement for a pheromone (Brennan and Zufall, 2006). It has also been

suggested that pheromones should not be consciously perceived. But the majority of pheromones will stimulate main olfactory receptors and therefore will have a perceptible odour so it would be more appropriate to state that pheromones do not have to be consciously perceived to have a pheromonal effect, as pheromonal receptors are typically several orders of magnitude more sensitive than canonical olfactory sensory neurons (Leinders-Zufall et al., 2000). One of the most useful refinements to the original definition of the term pheromone is the requirement for there to be mutual benefit to sender and receiver (Meredith, 1998), although this can be difficult to establish in practice. Built into this definition is the assumption that evolutionary selection has led to the co-evolution of the pheromonal signal and the pheromonal sensing system, with specialised receptors hard-wired to neural pathways eliciting an innate response. However, this does leave out a whole class of signals, such as individuality chemosignals that have evolved to transmit information about individual identity but that do not necessarily elicit an innate response and need to be learnt (Brennan and Kendrick, 2006). This requirement for learning means that they do not fit in the classical definition and they have been termed signature cues (Wyatt, 2010).

Innate vs learned chemosensory responses

The original definition of pheromonal action does not specify that responses need to be innate only that the responses should be “definite”. However, there are many general odour cues that have not evolved as specific signals that can be sensed and learned by the main olfactory system and it would not be useful to regard these as having pheromonal effects. Therefore, pheromonal signals are best regarded as mediating innate responses, i.e. they do not have to be learnt. Not all innate chemosensory responses are classified as pheromonal. Pheromone is the term given to cue acting within species. Cues acting between species, such as predator or prey cues are classed as allelochemicals (Wyatt, 2003), but may share similar sensory and neural pathways to pheromones. Examples of pheromonal responses in which the sensory receptors and neural pathways are most completely understood are those mediated by exocrine

secretory peptides (ESPs) in mice. These are a multigene family with around 20 members in mice encoding related 7kDa peptides that are sensed by the peptide/protein-sensing V2r class of vomeronasal receptors (Kimoto et al., 2005). Analysis of tissue expression levels of ESPS has identified two that are expressed in tear glands and sensed by the vomeronasal system following direct contact with the head region of the producer. The sex pheromone ESP1 is only produced by male mice, and constitutive knockout of the V2Rp5 receptor that mediates ESP1 action reduces lordosis quotient in female mice from 40% to 10% (Haga et al., 2010). ESP22 is produced by juveniles of both sexes and reduces sexual behaviour directed towards the juveniles by sexually mature males (Ferrero et al., 2013). Interestingly, lack of selective pressure on reproduction has led to significant differences in the pheromonal signals produced by different inbred strains of mice. For example males of the C57BL/6 strain lack production of ESP1 (Haga et al., 2010) and juveniles of the CBA strain produce very low levels of ESP22 (Ferrero et al., 2013). These differences between inbred strains are useful experimentally as they are effectively naturally occurring knockouts for these particular pheromones, but this also suggests that care needs to be exercised when investigating social behaviour using inbred strains of mice.

Another example of innate responses mediated by mammalian pheromones are the testosterone-dependent chemosignals present in urine from adult male mice that elicit aggression from other males and from lactating females. This aggression is elicited by both volatile and non-volatile constituents of male urine sensed by the vomeronasal organ (Chamero et al., 2007). The non-volatile constituents have been identified as a major urinary proteins (MUP). MUPs are lipocalins that bind small volatile ligands including brevicomin and thiazole, a mixture of which has also been found to elicit aggression, but only when added to the urine of castrated males (Novotny et al., 1985). This suggests that the brevocomin-thiazole mixture alone is insufficient to elicit aggression and needs to be sensed in the context of other, testosterone-independent urinary constituents to be effective. The context-dependence of this pheromonal effect is also evident in the requirement for a suitable conspecific that is associated with

the cues to act as a target for the aggression. When males sense the same urinary cues in the context of an unfamiliar urine mark encountered in their territory, countermarking behaviour is elicited rather than aggression (Humphries et al., 1999). Moreover, despite the fact that their own urine marks contain a similar mix of volatile and involatile chemosignals, they do not elicit countermarking behaviour presumably because they have been learned as being familiar (Hurst et al., 2001). Thus even though there are likely to be relatively direct and hardwired pathways from pheromonal input to behavioural output, these pheromonal effects are modulated by contextual cues and learning in mammals, as they are in invertebrates (Wyatt, 2014).

The aggression promoting effects of male urinary chemosignals are abolished by surgical ablation of the vomeronasal system (Clancy et al., 1984; Maruniak et al., 1986). The involvement of the vomeronasal system is further supported by the lack of aggression elicited by mature male intruders in the TRPC2 line of males and lactating females in which the gene for the vomeronasal transduction channel TRPC2 has been constitutively knocked out (Leypold et al., 2002; Stowers et al., 2002). This genetic manipulation produces a complex phenotype involving increases in inappropriately directed mounting behaviour in both males and females (Kimchi et al., 2007; Stowers et al., 2002). However, as was apparent from the earliest publications, TRPC2 knockout does not abolish all vomeronasal transduction (Kelliher et al., 2006; Leypold et al., 2002). TRPC2 is activated by the diacylglycerol branch of the phosphatidyl inositol bisphosphate signalling pathway. However, responses can still be generated by VSNs in TRPC2 knockout mice, by the release of intercellular Ca^{2+} via inositol trisphosphate signalling (Chamero et al., 2012) and subsequent activation of Ca^{2+}-dependent K^{+} and Cl^{-} channels (Dibattista et al., 2012; Kim et al., 2012). Different vomeronasal stimuli acting at different G-protein coupled vomeronasal receptors might differentially activate these two branches of the transduction pathway, leading to a selective deficit of specific vomeronasal stimuli in TRPC2 mice, while responses to other vomeronasal stimuli, such as MHC peptides is unimpaired (Kelliher et al., 2006). This can explain the various

discrepancies reported between the effects of vomeronasal ablation and TRPC2 knockout (Keller et al., 2009; Martel and Baum, 2009). Overall a picture is beginning to emerge that pheromones can not-only induce a specific behavioural reaction according to the original definition, but that some pheromonal effects can involve the inhibition of behaviours that would be elicited by other sensory cues in a context-dependent manner.

Although the vomeronasal system mediates many other pheromonal responses in mice, in addition to male aggression, it neither responds exclusively to pheromones, nor is it the only pheromonal sensing system. The mouse vomeronasal system mediates responses to a variety of urinary alleochemicals from predators, such as leopard and bobcat, by virtue of proteins from the MUP family secreted in their urine (Ben-Shaul et al., 2010; Papes et al., 2010). Also, pheromonal responses, such as nipple search conditioning to rabbit mammary pheromone (Distel and Hudson, 1985; Hudson and Distel, 1986), can be mediated by the main olfactory system and mouse alarm pheromones can elicit freezing and avoidance behaviour via the Gruneburg ganglion chemosensory system (Brechbühl et al., 2008).

The traditional view that the main olfactory system only mediates flexible, learned responses to volatile odours is beginning to change, as evidence accumulates that it is not a unitary system. Genetic ablation of main olfactory receptor neurons that project to the dorsal (D2) region of the main olfactory bulb (MOB) prevented innate aversion and freezing responses to the fox predator alleochemical trimethyltoluine (Kobayakawa et al., 2007). Furthermore, ablation of olfactory sensory neurons that projected to the D1 domain of the olfactory bulb prevented innate aversive responses to off-food odours, such as 2-methylbutyric acid. Importantly mice with these ablations could still respond to these odours and be conditioned to be attracted to them via receptors providing input to the ventral domain of the MOB (Kobayakawa et al., 2007). Thus there appear to be specialised chemosensory subsystems within the main olfactory system with certain dorsal regions of the MOB providing hard-wired input to central neural systems mediating either innate responses, via the bed nucleus of the stria

terminalis (Kobayakawa et al., 2007). Further evidence for the role of the main olfactory system in mediating innate responses has been provided by the discovery of the trace amine associated receptor (TAAR) family of olfactory receptors (Liberles and Buck, 2006). These are expressed by OSNs in the MOE and detect volatile amine stimuli, such as the putative mouse pheromone isoamylamine and the mouse predator alleochemical β-phenylethylamine which elicits stress and innate avoidance behaviour (Dewan et al., 2013). Other candidate main olfactory subsystems mediating innate responses include the OSNs that utilize the guanyl cyclase transduction mechanism that have been found to respond to atmospheric CO_2 levels (Hu et al., 2007). Also a subpopulation of OSNs that express the transduction channel TRPM5, which have been shown to have highly sensitive responses to pheromones such as dimethyl pyrazine and to MHC peptides, which are putative signature chemosignals (Lopez et al., 2014).

Signature chemosignals and learning

Although it is generally accepted that pheromonal responses are innate and not learned, there are evolutionarily-adapted chemosignaling systems in which the response is dependent on learning. These include the signature mixtures that have been proposed as a distinct class of chemosignal from pheromones (Wyatt, 2010). To be a reliable signal conveying individual identity, chemosignals need to be determined by an individual's genotype. This could be the result of individual differences in chemosensory profile that arise due to general heterozygosity in genetic makeup. But there are also polymorphic families of genes that have become specifically adapted to convey information about the genetic individuality of the producer. One such group of genes are those of the major histocompatibility complex (MHC). These encode MHC class I proteins that are expressed on all nucleated cells in the body and have evolved to enable self-non-self recognition by the adaptive immune system (Rammensee et al., 1995; Yamaguchi et al., 1981). Mice are capable of discriminating the odours of urine from congenic mice that differ genetically at only 3 amino acids in the binding groove of the MHC protein (Yamaguchi et al., 1981; Yamazaki et al., 1990). This

genetic difference is associated with a different profile of volatile odourants that can be sensed by the main olfactory system (Schaefer et al., 2001).

However, the profile of volatile constituents produced by an individual is liable to vary with physiological and environmental conditions such as food sources and microbial biota. An alternative individuality signal that is more directly related to MHC genotype are the peptide ligands that are naturally bound by the class I MHC proteins. These peptides are produced by proteosomal degradation of endogenous cellular proteins and foreign pathogens and are loaded onto into the binding groove of MHC class I proteins to be presented for immune surveillance a the cell surface. Crucially, peptide binding to MHC is critically dependent on the location of binding pockets in the binding groove, which accommodate bulky hydrophobic amino acid side-chains, known as anchor residues, at specific positions along the peptide sequence (Rammensee et al., 1995). The positions of these side-chains thus reflect the structure of the MHC class I peptide-binding groove and therefore the particular MHC genes expressed by a particular individual. Vomeronasal sensory neurons (VSNs) expressing V2rs have been found to respond to 9-amino acid peptides having a complementary structure to the MHC class I peptide-binding groove (Leinders-Zufall et al., 2004). Moreover, VSN responses were dependent on the location of the anchor residues along the peptide chain and thus reflected the MHC genotype of the producer. The VSN responses were also highly sensitive, responding down to concentrations as low as 10^{-14}M, as would be expected of a sensory receptive system that had adapted to detect the exceedingly low levels of MHC peptide ligands that would be expected to be released into the environment from bodily secretions (Leinders-Zufall et al., 2004).

The search for the existence of such MHC-dependent peptides in mouse urine has been impeded by the presence of complex mixture of urinary peptides at concentrations of around 10^{-7} to 10^{-8} M, many of which are produced by proteolytic cleavage in the kidney during the production of urine (Sturm et al., 2013). However, transgenic mice expressing the ovalbumin protein have been shown to excrete an ovalbumin-derived peptide at a concentration of around 10^{-}

^{12} M, which was absent from the urine of transgenic mice that lacked functional expression of MHC class I proteins (Sturm et al., 2013). In addition to MHC-dependent peptides, which convey the MHC genotype of the producer, Sturm et al found a large number of urinary peptides with single amino acid variations, produced by proteolytic degradation of endogenous proteins with natural single amino acid substitutions. These single amino acid variant peptides were effective stimuli for VSNs, providing an additional mechanism by which the general genetic heterozygosity of an individual could be conveyed by vomeronasal sensation (Sturm et al., 2013). Futhermore, a subpopulation of OSNs in the MOE have also been found to respond to peptide chemosignals (Spehr et al., 2006) suggesting the possibility that mice at least may be able to detect the same individuality chemosignals via both main olfactory and vomeronasal sensory systems. This finding also raises the possibility of the involvement of a peptide-based system of individuality recognition in animals, such as ungulates or carnivores that lack the V2r class of vomeronasal receptors, or in apes and other species in which the vomeronasal system is non-functional.

In mice (*Mus musculus*) and rats (*Rattus norvegicus*), the higher population densities due to their commensal lifestyle have led to the recent evolutionary expansion of the MUP gene family. Analysis of the mouse genome has revealed 21 functional genes encoding 18-20 kDa MUPs, which provide an additional basis for conveying individual chemosensory identity (Logan et al., 2008). MUP synthesis is developmentally and hormonally regulated. Although MUPs are produced by a number of secretory glands, including the salivary glands, lacrimal glands and mammary glands, the main site of production is the liver (Shahan et al., 1987). From there they are released into the blood plasma and secreted by the kidneys. Mouse urine contains high concentrations of MUPs, up to to 30 mg/ml in adult males, which produce three to four times as much as adult females (Beynon and Hurst, 2004).

MUPs play a vital role in mouse territorial behaviour. Being members of the lipocalin family, MUPs bind small volatile urinary constituents, such as (*R,* R)-3,4-dehydro-exo-brevicomin and (*S*)-2-sec-butyl-4,5-dihydrothiazole, which have

been identified as male urinary pheromones affecting female reproductive state and male aggression (Novotny, 2003; Novotny et al., 1999). The MUPs act as reservoirs for these chemosignals, prolonging their release from urine marks, (Hurst et al., 1998). Not only do the volatiles released by the MUPs attract both male and female attention to the urine marks, but also the amount of volatiles being released effectively signals the relative age of the urine mark (Hurst and Beynon, 2004). Moreover, the MUPs themselves, when stripped of their bound ligands have been found to have pheromonal activity in eliciting countermarking behaviour by males. However, a male mouse does not countermark in response to his own urine marks and this recognition of the individuality of the urine mark is conveyed by the profile of MUP variants it contains (Hurst et al., 2001). Urine from an individual male mouse contains between 5-15 different MUPs and each individual in a wild population produces a unique MUP profile (Robertson et al., 1997). This MUP profile in the urine marks can be used by both males and females to assess the competitive ability of males and plays an important role in inbreeding avoidance (Sherborne et al., 2007). A further source of individuality chemosignals derived from the MUP expression profile is provided by proteolytic cleavage of MUP proteins into peptides that can potentially be sensed by V2rs (Sturm et al., 2013). However it should be remembered that the individuality signalling systems that have been identified in mice are highly adapted and are not typical of other mammalian species. For instance, even the closely related aboriginal mouse species *Mus macedonicus* possesses a single MUP variant and therefore lacks the extensive MUP diversity found in *Mus musculus* (Robertson et al., 2007).

There are thus multiple types of signature chemosignals, related to MHC gentotype, MUP profile, genetic heterozygosity and volatile odour profile, all of which can potentially signal the individual identity of the producer. But the response they elicit is dependent on learning. Individual types of chemosignal may convey individuality in specific contexts, such as MUPs in territorial marking (Hurst and Beynon, 2004) and MHC peptides in mate recognition (Leinders-Zufall et al., 2004). However, it is likely that learning associates the information

provided by these different systems into a unified representation of individual chemosensory identity mediated by volatile and non-volatile cues sensed by both the main olfactory and vomeronasal systems.

Pheromonal conditioning

In addition to eliciting direct effects on behaviour or physiological state, pheromones can also act as unconditioned stimuli, causing the learning of non-pheromonal cues. One of the best examples of this is the rabbit mammary pheromone that has been identified as primarily being mediated by 2-methylbut-2-enal (2MB2) present in areolar secretions from the skin around the nipple of lactating rabbits (Schaal et al., 2003). Rabbit mammary pheromone elicits a characteristic behavioural arousal and nipple search behaviour in neonatal rabbits that is likely to be mediated by the main olfactory system (Hudson and Distel, 1986). This innate response is observed in caesarean-delivered neonates prior to their first sucking experience and enabling neonates to locate a nipple and is vital for successful suckling and reproductive success (Distel and Hudson, 1985). Although nipple search behaviour is primarily a response to the mammary pheromone, neonatal rabbits can also be conditioned to respond with nipple search behaviour to non-pheromonal odours. For instance, if the ventrum of the doe has been painted with the artificial odour, a single 5-minute suckling experience on the scented doe is sufficient for robust nipple search behaviour to be elicited by subsequent exposure to that odour (Hudson, 1985).

Although it might be supposed that this conditioning is likely to be explained by the reinforcing effects of milk acting as an unconditioned stimulus, this appears not to be the case. Rabbit neonates that receive a single paired 5-minute exposure to the mammary pheromone 2MB2 and an artificial odour subsequently show robust nipple search behaviour in response to exposure to the artificial odour alone (Coureaud et al., 2006). Thus in addition to acting as a releaser pheromone to elicit nipple search behaviour, the mammary pheromone is itself is acting as an unconditioned stimulus that induces learning to any odours with which it is paired. This has obvious adaptive significance in that conditioning to non-pheromonal components of maternal odour will reinforce the behavioural

response to the mammary pheromone and may help to maintain suckling following the decline in the doe's mammary pheromone production prior to weaning.

Certain pheromones sensed by the vomeronasal system also appear to be innately rewarding, with lesions of the vomeronasal system leading to extinction of attraction to sexual signals (Beauchamp et al., 1985; Oboti et al., 2014). Female mice show an innate preference to investigate adult male urine rather than female or castrated male urine (Moncho-Bogani et al., 2002). Attraction was only observed when females are allowed direct contact with male urine-soiled bedding, suggesting that naïve female mice have no innate preference for male urinary volatiles (Moncho-Bogani et al., 2002; Oboti et al., 2014; Ramm et al., 2008). Lesions of the AOB abolished this innate preference implying that the attractive chemosignal is an involatile, testosterone-dependent component of male urine that is being sensed by the vomeronasal system (Martinez-Ricos et al., 2008). However, these findings are contradicted by studies on oestrogen and progesterone treated, oviariectomised mice, which have observed innate attraction to male urinary volatiles (DiBenedictis et al., 2012). Whether such discrepancies are due to prior chemosensory exposure of the females, or differences in oestrous state remains to be determined.

Direct physical contact with male urine not only elicited an attraction to the urine, but also induced learning of the associated volatile urine odours and a conditioned place preference for the context in which the urine exposure occurred (Martinez-Ricos et al., 2007). Importantly, experiments using more genetically heterogeneous wild-derived mice have shown that the odour conditioning is to the specific volatile profile of an individual male rather than a more general odour of maleness (Ramm et al., 2008). Analysis of the protein constituents of male urine has revealed that both the sexual attraction effect and the odour and place conditioning effects are mediated by an atypical 18kDal MUP named darcin (Roberts et al., 2012b; Roberts et al., 2010). Urine from males of the BALB/c strain that have very low endogenous production of darcin lack both attraction and conditioning effects. But addition of recombinant darcin to

BALB/c male urine is sufficient to elict both effects (Roberts et al., 2010). As in the case of the rabbit mammary pheromone, a conditioned attraction to male volatile odours reinforces the innate attraction effect of male urine mediated by darcin. But more importantly, it associates both the volatile and non-volatile chemosensory profile of the individual male that produced the urine mark with its environmental location. This ability underlies the ability to use urine marks to judge the competitive ability of a male mouse.

The medial amygdala integrates vomeronasal and main olfactory information

A simple hypothesis to explain darcin-mediated odour conditioning would be for convergence of inputs from the main olfactory and vomeronasal inputs onto the same postsynaptic neurons (fig. 1). In naïve animals the innate pheromonal response would be mediated by the vomeronasal pathway. During exposure to the pheromonal signal, Hebbian plasticity of the main olfactory input onto the neurons activated by pheromonal stimulation could strengthen the connections leading to subsequent effectiveness of the learned main olfactory input to activate the innate pathway in the absence of vomeronasal input. The vomeronasal and main olfactory pathways are segregated at the level of the olfactory bulb. Therefore the first site at which direct integration of vomeronasal and main olfactory information could occur is likely to the corticomedial amygdala.

Such convergence in the medial amygdala has been suggested to account for the effects of sexual experience on mating in male hamsters. Sexually inexperienced male hamsters in which vomeronasal input was removed showed significant impairment of mating behaviour (Meredith, 1986). However, there was little impairment of mating behaviour in males in which vomeronasal ablation occurred after an initial mating experience, suggesting that mating behaviour was initially driven by innate pheromonal signals sensed by the vomeronasal system, but main olfactory cues learned at the first mating experience were sufficient to subsequently drive the behaviour in the absence of vomeronasal sensation (Meredith, 1998). However, the situation is complicated by the finding that

mating in sexually-naïve hamsters is not completely dependent on vomeronasal stimulation. Naïve hamsters with vomeronasal ablation have been found to mate normally following a priming exposure to hamster vaginal fluid forty minutes prior to mating (Westberry and Meredith, 2003). The proposed explanation for this is that a chemosignal in hamster vaginal secretions and sensed by the main olfactory system, has a priming effect on central mating circuits, which would normally be driven effectively only by vomeronasal input. This priming would increase their sensitivity allowing them to be driven by input from the main olfactory system (Westberry and Meredith, 2003). Although these findings may not generalise to other species, such as mice (Pankevich et al., 2004), they do indicate a role for experience and context in modulating pheromonal effects.

Different sub-regions of the medial amygdala respond to different categories of chemosensory signals, as assessed by c-Fos immediate early gene expression following chemosensory exposure. The anterior medial amygdala of mice appears to respond to a range of different categories of conspecific social odour stimulation from conspecifics and alleochemicals from heterospecifics (Samuelsen and Meredith, 2009). While the posterior medial amygdala shows segregation of responses to conspecific stimuli, including opposite sex stimuli, in the posterodorsal medial amygdala and to heterospecific stimuli, such as predator odours, in the posteroventral medial amygdala. These con-specific and heterospecific-responding regions of the amygdala provide input to hypothalamic regions controlling reproductive and defensive/aggressive behaviours respectively (Choi et al., 2005). Although VSNs expressing the V1r class of vomeronasal receptor have highly selective responses to individual chemosignals (Leinders-Zufall et al., 2000), the chemosignals are not necessarily specific to an individual category of producer. Hence V1r-expressing VSNs frequently are found to respond across more than one category (Isogai et al., 2011). In contrast, V2r-expressing VSNs respond more selectively to individual categories, with largely non-overlapping responses to male and female conspecifics, as well as to different predator and non-predator heterospecifics (Isogai et al., 2011).

There is therefore the potential for a relatively hard-wired labelled-line coding of vomeronasal information, linking specific stimuli directly to stereotyped responses. However, AOB mitral cells can sample input from more than one vomeronasal receptor type, at least as far as the V1r-expressing VSNs are concerned (Wagner et al., 2006). Hence in vivo electrophysiological recordings of AOB mitral cell activity in anaesthetised mice found that 31.4% of responding mitral cells responded exclusively to predator urine, 26.3% responded exclusively to conspecific urine and the remaining 39.3% responded to both predator and conspecific urine (Ben-Shaul et al., 2010). It is therefore likely that chemosensory stimulation at the level of the AOB is at least partly represented combinatorially in the pattern of mitral cell activation, rather than purely as a labelled line system that might be expected in a system adapted to mediate innate pheromonal responses.

There also appears to be little evidence for a labelled line system in the pattern of projections of AOB mitral cells to the amygdala. Anterograde, dextran tracing from small injections labelling as few as 50 mitral cells in either anterior or posterior sub-regions of the mouse AOB result in labelling of the input layer throughout the entire extent of the medial amygdala (von Campenhausen and Mori, 2000). This suggests a distributed mitral cell input onto medial amygdala neurons similar to the distributed projection of MOB mitral cells to piriform cortex in the main olfactory system (Haberley, 1985). Moreover, the mouse medial amygdala also receives a direct projection from mitral cells of the ventral main olfactory bulb, terminating in a more superficial input layer overlapping with AOB input throughout the anterior and posterodorsal medial amygdala (Kang et al., 2009). Individual neurons in the medial amygdala are therefore ideally positioned, not only to integrate information from different sub-regions of the AOB across different categories of vomeronasal stimuli, but also with information about volatile odours and peptides via the main olfactory system.

These convergent inputs from AOB and MOB makes their synaptic inputs onto medial amydala neurons a potential site for the association of main olfactory inputs with vomeronasal outputs that underlies the odour conditioning effects of

darcin on female mice and main olfactory associations formed at mating in male hamsters. Recently we have observed classical long-term potentiation of these input synapses in saggital medial amygdala slices *in vitro*, demonstrating the potential for plasticity at these synapses (unpublished observations). Moreover, oxytocin enhanced long-term potentiation produced by sub-threshold tetanic stimulation, reflecting the importance of oxytocin in the medial amygdala in both social recognition (Ferguson et al., 2001) and the response to chemosensory stimulation in general (Samuelsen and Meredith, 2011). However, the medial amygdala also receives indirect input from the main olfactory system via intra amygdala connections from anterolateral cortical areas, as well as a number of extra amygdalar inputs, for example from the BNST. Plasticity of any of these synaptic inputs could also provide a basis for association of main and vomeronasal chemosensory information (Shindou et al., 1993).

Gating of pheromonal responses

Although pheromonal responses are generally regarded as being innate and relatively stereotyped, they are still dependent on context. For example, a male mouse sensing the urine of a competitor male in a urine mark elicits countermarking behaviour (Humphries et al., 1999), whereas the same urine stimulus when painted on the fur of a castrated intruder male elicits aggression (Chamero et al., 2007; Mugford and Nowell, 1970). Many insect pheromonal effects are known to be modulated by hormonal state, and so it is not surprising that activity in the vomeronasal pathway has also been shown to be dependent on endocrine factors (Wyatt, 2014). For instance, electrical stimulation of the accessory olfactory bulb in female mice, was found to be more effective in driving tuberoinfundibular arcuate hypothalamic neurons in the presence of oestrogen (Li et al., 1989). This suggests that pheromonal input alone is insufficient to drive behavioural outputs in the absence of the appropriate context, or that environmental context or physiological state is able to gate the activation of specific pheromonal responses. For example, gating of the transmission of chemosensory information has been proposed to explain mate recognition

memory in mice (Brennan et al., 1990). This memory is formed by female mice to the male's signature mixture that she is exposed to during a sensitive period immediately following mating (Keverne and de la Riva, 1982; Rosser and Keverne, 1985). Recognition of her mate's signature mixture during subsequent exposure prevents them from eliciting the pregnancy blocking effect that normally occurs in response to exposure to male urinary chemosignals during the pre-implantation period (Bruce, 1959).

This pregnancy block (Bruce effect) is elicited by exposure of recently-mated female mice to testosterone-dependent male chemosignals, coincident with the twice-daily prolactin peaks that occur following mating (Rosser et al., 1989). Pregnancy blocking effects of male exposure have been observed in a limited number of other species, including prairie voles and wild geladas (Fraser-Smith, 1975; Roberts et al., 2012a). However, in these species it is unclear whether these effects are mediated by chemosensory cues and therefore by similar neural mechanisms to the pregnancy block effect in mice. In mice, the Bruce effect is mediated by the vomeronasal system, as pregnancy block is prevented by ablation of the vomeronasal organ, but unaffected by ablation of the main olfactory epithelium (Lloyd-Thomas and Keverne, 1982; Ma et al., 2002). Pregnancy block is elicited by activation of a neural pathway, via the AOB and corticomedial amygdala, ultimately stimulating dopamine release by tuberoinfundibular dopaminergic neurons of the arcuate nucleus of the hypothalamus (Li et al., 1989, 1990). This in turn suppresses prolactin release from the anterior pituitary gland, removing luteotrophic support and resulting in a decline in progesterone production by the corpora lutea and a return to oestrus (Dominic, 1966). Mate recognition can be explained simply by a gating of this pheromonal response, preventing activation of the activation of the arcuate neuroendocrine output (Brennan and Kendrick, 2006).

Although the prevalence and importance of the Bruce effect in wild mice remains to be determined, it has been studied extensively in laboratory experiments using different inbred strains as the mating male and unfamiliar, pregnancy-blocking male. Both the pregnancy blocking effectiveness and the

signature mixture underlying individual recognition are conveyed by low molecular weight (<5kDa) constituents of male urine (Peele et al., 2003). Urine from a male of the BALB/c inbred strain, is normally ineffective in blocking pregnancy following mating with a BALB/c male. However, pregnancy blocking effectiveness can be conferred by the addition to the BALB/c urine of nine-amino acid MHC peptide ligands of the type that would normally be bound by MHC class I proteins of an unfamiliar male of the C57BL/6 strain (Leinders-Zufall et al., 2004). These experiments suggest that the individuality signal underlying mate recognition is based on the MHC type of the male and conveyed by MHC peptide ligands, although the urinary constituents responsible for the pregnancy block effect itself remain to be identified. The MHC basis for mate recognition would enable recognition of different individuals in a wild population, although the incidence and importance of the pregnancy effect in natural contexts is not well understood.

Gating of the Bruce effect by mate recognition

A large body of research over the last thirty years has focused on understanding the neural mechanisms involved in mate recognition memory formation. The mechanisms appear to differ from those underlying episodic memories as mate recognition is unaffected by hippocampal lesions (Selway and Keverne, 1990). Attention has therefore focused on identifying a locus for memory formation within the vomeronasal pathway itself. This cannot be studied using classical lesioning techniques, as any physical disruption of the vomeronasal pathway that might affect memory formation would also prevent the pregnancy block response. Instead, local infusions of the anaesthetic lignocaine have been used to temporarily inhibit neural transmission at different stages of the vomeronasal pathway immediately following mating, during the sensitive period for memory formation. Infusions of lignocaine into the AOB prevented memory formation, as might be expected. However, infusions of lignocaine in the projection sites of the AOB in the corticomedial amygdala failed to prevent memory formation (Kaba et al., 1989). This suggests that synaptic plasticity within the AOB is necessary and sufficient for memory formation. Subsequently, memory formation has been

prevented by a range of pharmacological interventions directed at the AOB (fig. 2). Memory formation was inhibited by AOB infusions of the PKC inhibitor polymyxin, over a 4.5-hour period following mating, and by the protein synthesis inhibitor anisomycin during a later period 3-6 hours post mating (Kaba et al., 1989). The AOB has particularly high levels of nitric oxide synthase, and although AOB infusions of nitric oxide synthase inhibitors do not prevent memory formation (Brennan and Kishomoto, 1993), AOB infusions of nitric oxide donors enhance memory formation (Okere et al., 1996), consistent with the memory-enhancing role of nitric oxide signalling in other neural systems.

The differential effects of local infusion of ionotropic glutamate receptor antagonists on memory formation provide further evidence that synaptic plasticity in the AOB is not only necessary, but also sufficient to explain mate recognition memory in mice. AOB infusions of the non-selective antagonist gamma-D-glutamylglycine (DDG) or the selective NMDA receptor antagonist D-2-amino-5-phosphonovaleric acid (APV) or the selective AMPA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) all cause a direct pregnancy block due to dis-inhibition of AOB mitral cell activity resulting from a reduction in activation of granule cell inhibitory interneurons (Brennan, 1994; Brennan and Keverne, 1989). Thus all 3 of these antagonists are likely to have similar effects downstream from the AOB to activate the neuroendocrine pregnancy blocking output. However, the common, pregnancy blocking effect of these drug infusions is dissociated from their different effects on memory formation. Non-selective antagonism of ionotropic glutamate receptors with DDG, or a combination of APV and DNQX, prevented memory formation, whereas infusions of APV or DNQX alone did not (Brennan, 1994; Brennan and Keverne, 1989). Moreover, infusions of DNQX actually promote the formation of a “global” memory in the absence of mating, presumably by the intense stimulation of NMDA receptors on granule cells, as a result of the mitral cell disinhibition. This dissociation of AOB mitral cell disinhibition and memory formation suggests that the neural mechanisms of the induction of mate recognition memory formation are intrinsic to the AOB.

The formation of mate recognition memory is associated with substantial, electrophysiological, neurochemical and morphological changes in the AOB. Following mating there was a 200-300% increase in the power of the oscillatory local field potential recorded in the granule cell layer of the AOB of awake mice. Moreover, there was also a differential local field potential power in response to the mating compared to unfamiliar male chemosignals (Binns and Brennan, 2005). This could be explained either by a greater synchronisation of neural activity and/or potentiated synaptic transmission in response to mating male chemosignals. Microdialysis measurements from awake mice revealed increased levels of the inhibitory neurotransmitter GABA in the AOB of mated females during exposure to the mating male chemosignals (Brennan et al., 1995). As the GABA is being released predominantly by granule cells, this suggests that subpopulation of AOB mitral cells responding to the mate's chemosignals become more effective in activating the inhibitory granule cell interneurons following mating. Furthermore, there is a selective increase in the length of the excitatory synapse from mitral to granule cells in the AOB following mating (Matsuoka et al., 2004). All these lines of evidence point to an increase in the inhibitory gain of the reciprocal synapses between mitral and granule cells as a key mechanism for memory formation. This is backed up by *in vitro* recordings from mouse AOB slices, which have demonstrated NMDA receptor-dependent long-term potentiation of synaptic transmission at the mitral to granule cell side of the reciprocal synapse (Kaba and Huang, 2005).

These findings, are consistent with a simple hypothesis for mate recognition memory formation (Brennan et al., 1990; Kaba and Nakanishi, 1995). It is known that AOB mitral cells respond to specific combinations of sex and strain of an anaesthetised conspecific (Luo et al., 2003). It is therefore hypothesised that the subpopulation of mitral cells that respond to the mating male chemosignals, and are therefore activate at the time of mating, undergoes NMDA receptor-dependent long-term potentiation at their glutamatergic synaptic input to granule cells. Subsequent exposure to the same male chemosignals, during the vulnerable pre-implantation period, would thus be more effective at

exciting granule cells, via the potentiated synapses. The mitral cells responding to the mate's chemosignals would consequently receive enhanced feedback inhibition from granule cells at the reciprocal synapses. This selective enhancement of self-inhibition would gate the transmission of the mating male's chemosensory signal, preventing it from activating the neuroendocrine pregnancy block response (fig. 3).

The final neuroendocrine output of the pregnancy block pathway is via activation of dopaminergic neurons in the arcuate hypothalamus, which inhibits prolactin release from the anterior pituitary (Li et al., 1990). Exposure of a mated female to soiled bedding from an unfamiliar strain of male increased the expression of the neural activity marker c-Fos in these neurons. In contrast, exposure to soiled bedding from the mating male failed to increase c-Fos expression these arcuate dopaminergic neurons (Matthews et al., 2013). This evidence is consistent with a gating of the mating male's chemosignals following mating that prevents them from activating the neuroendocrine pregnancy block output. Similar evidence for a suppression of c-Fos activation in response to the mating male chemosignals has been reported at earlier levels of the vomeronasal pathway, including the anterior and the posterodorsal medial amygdala, along with the bed nucleus of the stria terminalis and medial preoptic area of the hypothalamus (Halem et al., 2001). Although a subsequent study has failed to reproduce this finding (Matthews et al., 2013). Nevertheless, neurons in the medial amygdala were found to respond equally well to both mating and unfamiliar male urine applied to the nose of unmated females. Whereas neurons in the medial amygdala of mated females fired half as many spikes in response to the mating male urine compared to urine from an unfamiliar strain of male (Binns and Brennan, 2005).

Overall, the experimental evidence supports a suppression of responsiveness to mating male chemosignals at sites along the vomeronasal pathway downstream from the AOB. But there is no evidence for a differential expression of c-Fos of mitral cells at the level of the AOB as might be expected if the mitral cells responding to the mating male were subject to the hypothesised

enhanced inhibitory feedback from granule cells (Halem et al., 2001; Matthews et al., 2013). One reason for this might be the sparseness of the sub-population of mitral cells that respond to the mating male, leading to a small effect size. However, it is also possible that increased inhibitory feedback from granule cells changes the timing of mitral cell spike activity in response to the mating male rather than simply inhibiting it. A change in the intrinsic frequency of mitral cell activity could effectively decouple oscillatory neural activity in the AOB from the intrinsic oscillatory mode of downstream vomeronasal brain areas (Taylor and Keverne, 1991). This could decrease the effectiveness of the mitral cells in activating the central brain areas on the pregnancy blocking pathway, without significantly affecting their firing rate. Although dramatic changes in the amplitude of AOB local field potential oscillations have been observed following mating, across a range of frequencies (Binns and Brennan, 2005), it remains to be established whether the coherency of these oscillations with those in central vomeronasal areas is actually affected by learning.

Neuromodulation of AOB circuits

Mate recognition memory formation is contingent on mating. Simple exposure to male chemosignals without mating does not result in subsequent male recognition (Keverne and de la Riva, 1982). Therefore the question arises as to how mating is signalled to the AOB and how it induces plasticity at active reciprocal synapses. The prime candidate for this mating signal is an increase in the release of noradrenaline from the locus coeruleus neuromodulatory system. The rodent olfactory bulb is known to receive a particularly dense projection of noradrenergic fibres from the locus coeruleus (Shipley et al., 1985), and enhanced noradrenaline release within the olfactory bulb is thought to underlie the formation of social odour memories in a variety of contexts (Brennan and Kendrick, 2006).

Following mating, expression of the activity marker c-Fos are increased selectively in a small sub-population of noradrenergic neurons in the locus coeruleus, which were also retrogradely labelled by fluorescent microbeads injected into the AOB (unpublished observations). Moreover, *in vivo* microdialysis

in awake female mice has revealed that the concentration of noradrenaline in the AOB was significantly increased during the sensitive period for memory formation following mating (Brennan et al., 1995). Depletion of olfactory bulb noradrenaline following local injections of the catecholaminergic neurotoxin, 6-hydroxy-dopamine six days prior to mating prevents memory formation to the mating male chemosignals (Rosser and Keverne, 1985). Moreover, local infusions of the α-ADR antagonist, phentolamine, but not the β-ADR antagonist, propranolol, during the sensitive period prevent memory formation (Kaba and Keverne, 1988).

This dependence of memory formation on noradrenergic transmission in the AOB is further supported by *in vitro* studies. Noradrenaline was found to enhance the LTP produced by subthreshold mitral cell theta stimulation in mouse AOB slices. Furthermore, LTP at the mitral to granule cell synapses was blocked by the α_2 adrenergic antagonist idazoxan, but not by the α_1 antagonist prazosin, or the β antagonist propranolol (Kaba and Huang, 2005). Interestingly, α_2 adrenergic transmission has also been shown to induce plasticity in MOB slices *in vitro*. Pairing bath applied noradrenaline or α_2 adrenergic agonist with stimulation of the olfactory nerve input resulted in a lasting increase in the power of gamma-band LFP oscillatory activity of around 200-300% (Gire and Schoppa, 2008; Pandipati et al., 2010), similar to that observed in the AOB *in vivo* (Binns and Brennan, 2005; Leszkowicz et al., 2012).

Mating mice come together for numerous bouts of intromission, prior to ejaculation, at which point the ejaculate solidifies to form a plug within the vagina. It is hypothesised, therefore, that this drawn out process facilitates enhanced NA release within the AOB that acts via α-ADR to modulate a plastic change within the AOB to alter neuronal network activity. However, the precise mechanism by which noradrenaline induces synaptic plasticity is unclear. α_2 receptors have been shown to decrease presynaptic Ca^{2+} currents via N-type Ca channels in mitral cells in AOB slice preparations (Dong et al., 2009). This would be expected to have a disinhibitory effect on mitral cell activity. Interestingly mGluR2 receptor stimulation also decreases presynaptic Ca^{2+} currents in both mitral and granule cells and leads ultimately to disinhibition of mitral cells (Dong et al., 2009;

Hayashi et al., 1993). Local infusions of the mGluR2 agonist have been found to induce memory formation in the absence of mating (Kaba et al., 1994). Noradrenaline acting via α_2 adrenergic receptors may therefore act synergistically with glutamate release from mitral cells at mGluR2 receptors to disinhibit mitral cells resulting in potentiation of their synapses with granule cells.

Such a mechanism is consistent with the finding that artificial vagino-cervical stimulation of anaesthetised female mice caused a disinhibition of AOB mitral cell firing in around 50% of mitral cells recorded in anaesthetised mice (Otsuka et al., 2001). However, the mechanisms of action of both noradrenaline and glutamate in the AOB are complex, involving both increases and decreases in firing rate and likely to depend on spatiotemporal patterns of noradrenaline release and on the cellular distribution of the adrenergic receptor subtypes (unpublished observations). Stimulation of α_1 adrenergic receptors and mGluR1 receptors, in mouse AOB slices in vitro, has been found to increase the release of GABA from granule cells consequently increase inhibition of mitral cells (Araneda and Firestein, 2006; Smith et al., 2009). Furthermore, infusions of noradrenaline into the AOB of awake mice resulted in a lasting increase in the power of the AOB LFP oscillation, similar to that observed after mating, but without causing any significant post-infusion disinhibition of mitral cell activity (Leszkowicz et al., 2012).

Thus the mechanism for noradrenaline action in the AOB is complex and consistent with two hypotheses. Noradrenaline may act to increase signal to noise ratio in the AOB, suppressing activity in the majority of mitral cells through action at α_1- adrenergic receptors, whilst simultaneously enabling increased activity of mitral cells that respond to the mating male, via α_2- adrenergic receptors, in concert with mGluR2 mediated disinhibition. Alternatively, a two stage process has been proposed (Dong et al., 2009). According to this hypothesis, the enhanced noradrenaline release at mating would first activate the higher affinity α_2-adrenergic receptor, which along with mGluR2 receptor activation, would result in an initial mitral cell disinhibition of the subset of mitral cells responding to the mating male’s chemosignals. Following this, a slower

activation of lower affinity α_1-adrenergic receptors and group I mGluRs in combination with enhanced intracellular Ca^{2+} levels, following M/T cell disinhibition, could lead to the activation of the α isoform of PKC, allowing a rebound enhancement of granule cell activity and subsequent inhibition of mitral cell firing (Dong et al., 2009).

Levels of the neuropeptide oxytocin are also increased at mating and have been proposed to play a role in mate recognition memory formation. Oxytocin knockout mice are unable to recognise their mate following mating (Wersinger et al., 2008). Furthermore, oxytocin facilitates LTP at the mitral to granule synapse in mouse AOB slices *in vitro* (Fang et al., 2008). This dependence of mate recognition memory formation fits into a wider role for neuropeptides such as oxytocin and vasopressin in the modulation of social behaviour. Oxytocin knockout mice have impaired social recognition, which can be rescued by oxytocin infusion into the medial amygdala, but not the olfactory bulb (Ferguson et al., 2001; Ferguson et al., 2000). However, there may be a species difference in oxytocin effects, as oxytocin infusions into the MOB have been found to prolong the duration of social memory in rats (Dluzen et al., 1998). Notably, this effect was mediated by stimulating the release of noradrenaline in the MOB, and was dependent on α_2 adrenergic receptors (Dluzen et al., 2000). Whether a similar dependence on increased α_2 adrenergic transmission in the mouse AOB underlies the dependence of mate recognition memory formation on oxytocin remains to be determined.

A role for the main olfactory system in mate recognition?

The pregnancy blocking effect of unfamiliar male chemosignals, during the pre-implantation period of recently mated females, is prevented by vomeronasal organ lesions and is unaffected by ablation of the main olfactory epithelium (Lloyd-Thomas and Keverne, 1982; Ma et al., 2002). Nevertheless, although main olfactory input is not required for the pregnancy block effect, there is some evidence that the presence of the stud male has a general protective effect against pregnancy block to an unfamiliar male and to food deprivation (Archunan and Dominic, 1990; Kumar and Dominic, 1993). This is suggested to be by a

separate luteotrophic effect mediated by the main olfactory system rather than the selective gating of a luteolytic pregnancy blocking signal conveyed via the vomeronasal system (Archunan and Dominic, 1990). If such an effect does indeed occur it appears to also require memory formation to the mating male at the time of mating, but to differ in the memory having a duration of around 7 days following mating (Acharya and Dominic, 1997) rather than the 30 day duration of the mate recognition memory mediated by the vomeronasal system (Kaba et al., 1988).

It has also been reported that male chemosignals sensed by the main olfactory system can potentially block pregnancy during the post-implantation period. This is normally prevented by an increase in dopaminergic inhibition of olfactory sensory neuron by periglomerular cells, during the post-mating period, which selectively prevents social odours from activating the MOB (Serguera et al., 2008). However, this is based on the pregnancy blocking effects of male odour exposure following systemic treatment with dopaminergic antagonists. As dopaminergic periglomerular cells have also been reported to be present in the AOB (Matthews et al., 2013), these experiments need to be repeated targeting the MOB more selectively to confirm that main olfactory cues are indeed capable of eliciting a post-implantation pregnancy block. It would also be interesting to investigate whether the main olfactory mediated signals eliciting post-implantation pregnancy block involve activation of the vomeronasally mediated pregnancy block output via convergence at the level of the medial amygdala. The potential functional significance of this post-implantation pregnancy block is also unclear, given that it is normally gated by the post-mating increase in dopaminergic periglomerular cell inhibitory activity.

Neurogenesis and olfactory plasticity

The vomeronasal system and the main olfactory system are exceptional among sensory systems in that they undergo substantial neurogenesis and neuronal replacement in the adult mammal. At the peripheral level, both OSNs in the MOE and VSNs in the VNO undergo continual turnover, with a lifespan that depends both on establishing contact with their postsynaptic target and the damage

798 resulting from their high degree of environmental exposure (Schwob et al., 1992).
799 This continual replacement of sensory neurons from stem cells, provides the
800 possibility for clonal expansion of those expressing specific receptor types,
801 enabling adaptation of the sensory system to different physiological and
802 environmental contexts. There's good evidence that chemosensory cues
803 themselves can affect peripheral sensitivity. The proliferation and survival of
804 VSNs expressing the V2r class of vomeronasal receptor was found to be
805 enhanced by exposure to the MUP containing protein fraction of male mouse
806 urine, but not urine that had been stripped of protein (Xia et al., 2006). This
807 suggests a trophic role for MUPs, in addition to their role as sensory stimuli.
808 Responses of the V2r class of VSN that respond to MUPs could therefore be
809 optimised to detect the chemosensory cues from particular males that are in the
810 local environment. As such this mechanism has similarities with the selective
811 increase in peripheral sensitivity, following odourant exposure, observed in
812 electrolfactogram recordings from mouse MOE (Wang et al., 1993).
813 This potential for changes in peripheral sensitivity to mediate lasting
814 effects on chemosensory responses is highlighted by the effects of postnatal
815 exposure of mice to male urine during the first 18 days of life. This postnatal
816 exposure resulted in a behavioural preference for investigating the pre-exposed
817 urine as an adult, and was associated with epigenetic changes in expression
818 levels of both olfactory and vomeronasal receptors (Broad and Keverne, 2012).
819 Endocrine state is an additional factor that can affect the rate of turnover of
820 VSNs. The rate of VSN turnover was found to be increased in pregnant mice,
821 which may have a role in adapting the vomeronasal sensory systems to the
822 changes associated with parturition and maternal behaviour (Kaba et al., 1988).

823 Olfactory bulb neurogenesis and learning

824 Neurogenesis is not only a feature of peripheral chemosensory systems. The
825 olfactory bulb is one of the two structures in the mammalian brain that undergo
826 extensive neural turnover in the adult. Neurons and glia are continually being
827 born in the subventricular zone and migrate rostrally into the core of the olfactory
828 bulb in the rostral migratory stream (Luskin, 1993). By the time that the neurons

arrive in the olfactory bulb their fate has been specified as GABAergic interneurons and they migrate into the granule and glomerular layers of the MOB and AOB. It has been estimated that a thousand new interneurons reach the MOB daily and is balanced by a similar level of neuronal death (Imayoshi et al., 2008). After arriving at the MOB, there is a critical period of synaptogenesis between days 14 and 20 of neuronal development in which olfactory exposure can lead to the incorporation of the new neurons into active circuits in the mature olfactory bulb (Yamaguchi and Mori, 2005). Both neuronal survival and olfactory performance are enhanced in animals housed in an odour-enriched environment (Rochefort et al., 2002). Subventricular zone neurogenesis in female mice is enhanced by exposure to male chemosignals, an effect that is mediated by prolactin (Mak et al., 2007). Similarly, subventricular zone neurogenesis is also increased by the changes in prolactin levels associated with pregnancy and parturition (Shingo et al., 2003). There is substantial evidence linking neurogenesis with odour learning and discrimination. Thus these findings suggest that enhancement of chemosensory learning, as a result of enhanced neurogenesis, might enable chemosensory systems to adapt to different reproductive requirements.

Increased incorporation of newborn neurons into the MOB has been associated with odour discrimination learning (Alonso et al., 2006) and blockade of neurogenesis has been shown to prevent a learned improvement in an odour conditioning task (Sultan et al., 2010). However, this may depend crucially on the nature of the learning task, as simple odour association tasks appear to be unaffected by inducible genetic ablation of newborn neurons (Imayoshi et al., 2008; Sakamoto et al., 2011), while inhibition of neurogenesis using antimitotics prevents operant but not associative odour learning (Mandairon et al., 2011). The addition of new inhibitory interneurons to existing MOB circuits is likely to enhance the differentiation of the pattern of mitral cell activity in response to the learned odour from those produced by similar odours (Lepousez et al., 2013).

A similar role for neurogenesis may be involved in mate recognition learning in the AOB. Mate recognition memory formation in female mice was

prevented by long-term inhibition of neurogenesis by local infusions of anti-mitotics into the subventricular zone (Oboti et al., 2011). Interestingly, incorporation of newborn neurons into the AOB and mate recognition memory formation were also prevented by neurotoxic lesions of the medial amygdala. This suggests that incorporation of new neurons into the olfactory bulb may not only depend on direct chemosensory input but also on centrifugal input from central olfactory areas. However, this finding does appear to conflict with the finding that reversible silencing of the medial amygdala during the 6 hours following memory formation, using local anaesthetic infusions, failed to prevent memory formation (Kaba et al., 1989). It may be that the effect of centrifugal feedback from the amygdala in enhancing incorporation of newborn neurons occurs outside the post-mating sensitive period for the induction of memory formation in the AOB.

One caveat to the apparent dependence of mate recognition memory on addition of new neurons to the AOB is the finding that inducible genetic ablation of newborn neurons reduces the ability of female mice to maintain a pregnancy (Sakamoto et al., 2011). Surprisingly, genetic ablation of newborn neurons has been reported to result in a widespread impairment of innate behavioural responses mediated by the vomeronasal system. This includes impairment of aggression, but also to male sexual behaviour and maternal behaviour in females (Sakamoto et al., 2011). Whether innate behaviours in mice are indeed dependent on the incorporation of new interneurons to mature AOB circuits, or whether this effect is due to the inhibition of neurogenesis in another part of the vomeronasal pathway, such as the VSNs themselves, or neurons in the amygdala (Liu et al., 2014) remains to be determined.

Conclusions

Pheromones are generally regarded as mediating innate responses. However, this is difficult to determine in practice, as learned responses to chemosensory cues experienced in utero or early postnatal life may occur. If such learning occurs invariably, in all individuals of the same type in the course of normal

development, then the learned response is effectively indistinguishable from an innate response and can be classed as pheromonal (Wyatt, 2014). However, pheromonal effects can also be elicited by non-pheromonal stimuli as a result of learning, which plays a vital role in reinforcing the pheromonal response in a variety of contexts. Chemosensory cues that convey individual identity, such as MUPs or MHC peptides, also need to be learned to enable individual recognition. No one chemosensory system has a monopoly on pheromonal or kairomonal chemosensory responses. Moreover, associative learning enables the association of chemosensory information across chemosensory systems. Multiple sources of chemosensory information from peptides, proteins and volatile odourants can be associated at the level of the amygdala, which enables non-pheromonal cues to access innately-driven outputs. Plasticity in both the main olfactory system and the vomeronasal system can gate the transmission of pheromonal information to output circuits. This reflects a larger role for context dependence of pheromonal effects, which is more common than generally recognised by researchers in this field. Definitive responses to pheromones are most likely to be observed in the context of neonatal responses where the physical and social environment is relatively constant. But these are exceptions to the more general conclusion that mammalian pheromonal responses depend on both external context and internal factors such as endocrine state. One of the most interesting avenues for future research is the role of neurogenesis at both the level of the sensory neurons and central brain pathways in adapting chemosensory systems to the external environment and physiological priorities of the individual.

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Figure 1

Hypothesised role for convergence at the input synapses to the medial amygdala in pheromonally-mediated social odour conditioning in mice. Volatile constituents of male mouse urine are sensed by the main olfactory system and are conveyed via projections from the ventral main olfactory bulb (MOB). Involatile constituents of male mouse urine including individuality chemosignals and the attractant pheromone darcin are sensed by the vomeronasal system and conveyed via projections from the posterior accessory olfactory bulb (AOB). The MOB and AOB outputs project to overlapping regions of the input layer to the medial amygdala (MeA). Input from male urinary volatiles is insufficient to elicit attraction in naïve female mice. Exposure to darcin is hypothesised to strongly activate neurons in the MeA leading to behavioural attraction to the male urine. The strong activation of the MeA neurons is hypothesised to induce plasticity (indicated by *) at input synapses conveying information about both involatile and volatile individuality signatures. This learning may underlie formation of a “social chemosensory object”, which would enable subsequent recognition of an individual male on the basis of volatile and/or involatile chemosensory information. Subsequent exposure of the experienced female to the volatile individuality chemosignals from the same male are hypothesised to input via potentiated synapses and effectively drive activity in the MeA neurons, leading to behavioural attraction to the volatiles.

Figure 2

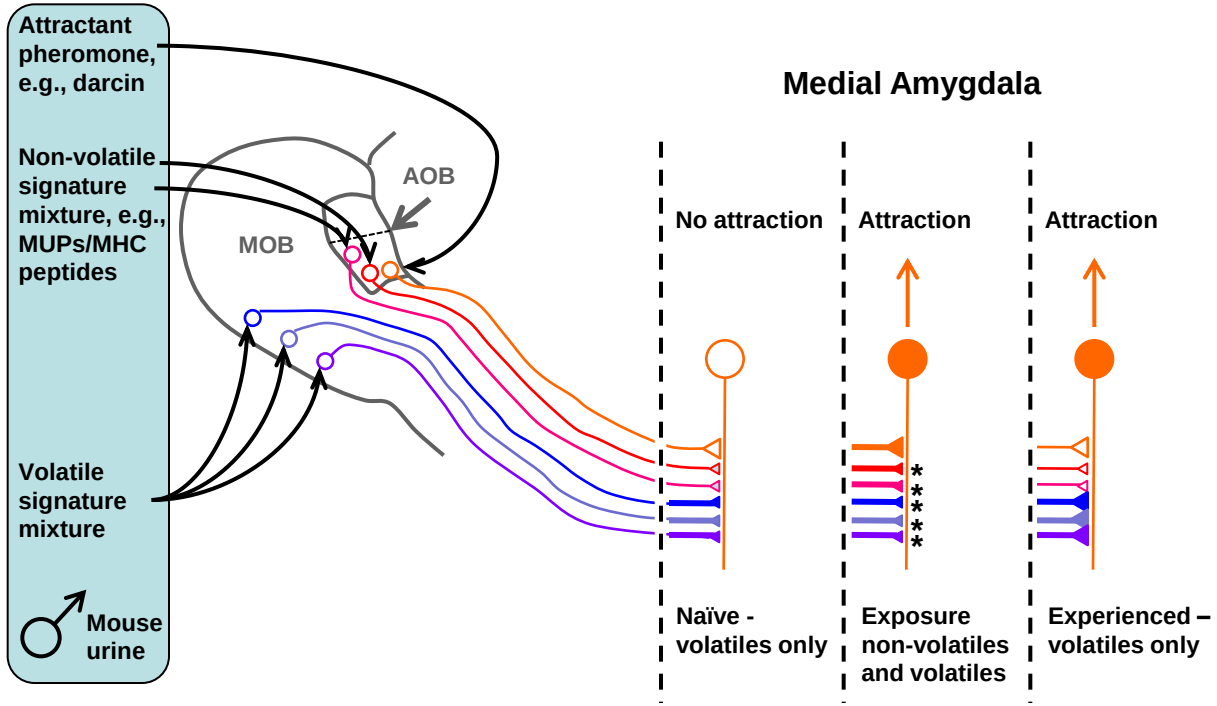
Neurochemical mechanisms and pharmacological interventions affecting mate recognition memory formation via their effects at the mitral/granule cell reciprocal synapse in the accessory olfactory bulb. Excitation of mitral cells by chemosignals from the mating male releases glutamate, depolarising granule cell spines. This results in release of gamma aminobutyric acid (GABA) from the granule cells to self-inhibit the mitral cells via a close-coupled negative feedback at the reciprocal synapse. Noradrenaline release at mating most likely acts via alpha2 adrenergic receptors, which along with activation of metabotropic glutamate (m2) receptors is thought to dis-inhibit mitral cells. Increased glutamatergic input to ionotropic glutamate receptors (iGluRs) induces synaptic potentiation, and memory formation, via an intracellular signalling pathway involving protein kinase C (PKC), nitric oxide synthase (NOS) and protein synthesis. The effect of local pharmacological interventions on long-term potentiation

(LTP) of the reciprocal synapse *in vitro*, or memory formation *in vivo* are shown. Abbreviations: APV, 2-amino-5-phosphonovaleric acid; DCG-IV, (2*S*,2'*R*,3'*R*)-2-(2',3'-Dicarboxycyclopropyl)glycine; DNQX, 6,7-dinitroquinoxaline-2,3-dione; Poly B, polymyxin B; SNP, sodium nitroprusside; VGCC, voltage-gated calcium channel.

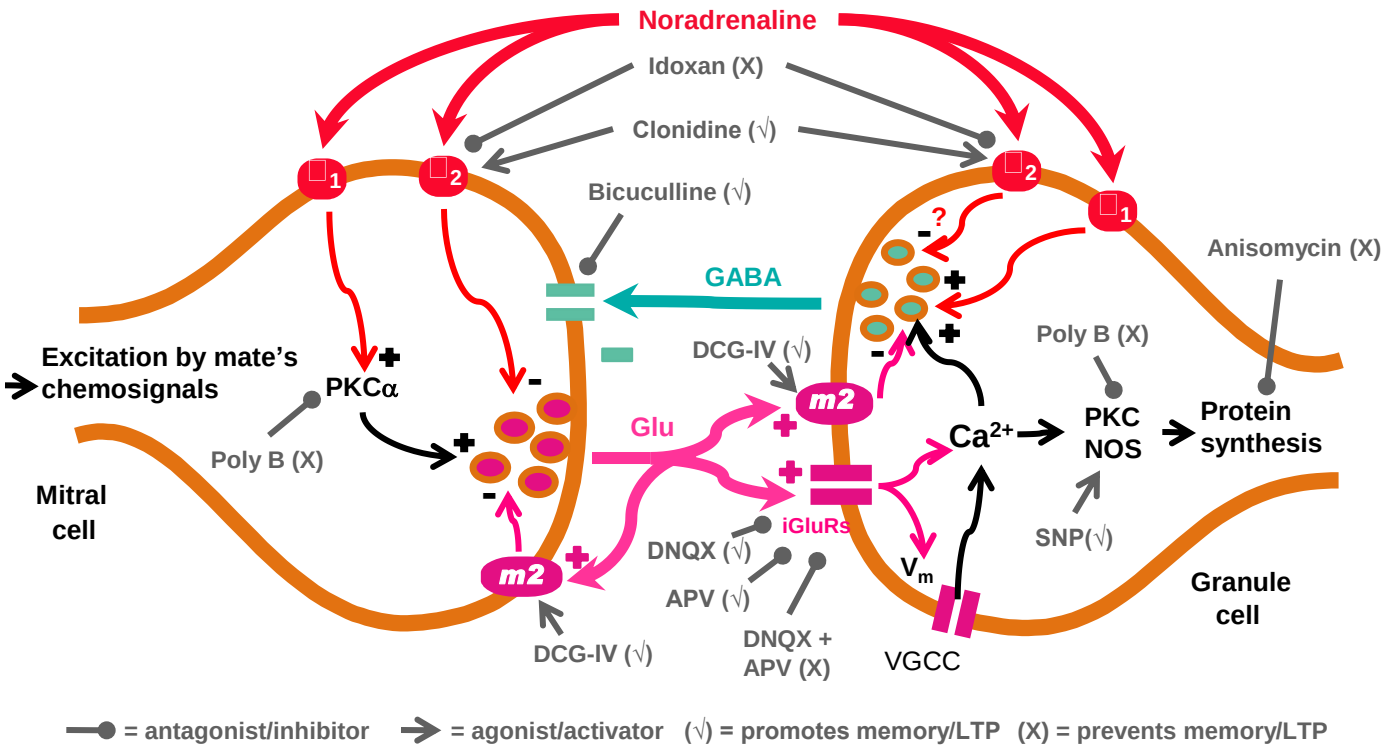
Figure 3

Gating hypothesis for the mechanism underlying mate recognition in the Bruce effect. (a) Before learning, the sub-population of accessory olfactory bulb (AOB) mitral cells excited by male chemosignals transmits the signal centrally to induce pregnancy block. (b) During learning, noradrenaline released into the AOB at mating induces long-term potentiation of mitral/granule cell reciprocal synapses that are activated by the mate's individual chemosignals. (c) After learning, re-exposure to the mating male chemosignals activates the sub-population of mitral cells with potentiated synapses. The increased inhibitory gain of these synapses prevents activity in the subpopulation of mitral cells that respond to the mating male's chemosignals from being transmitted centrally, preventing pregnancy block. Abbreviations: PG, periglomerular; VNN, vomeronasal nerve.

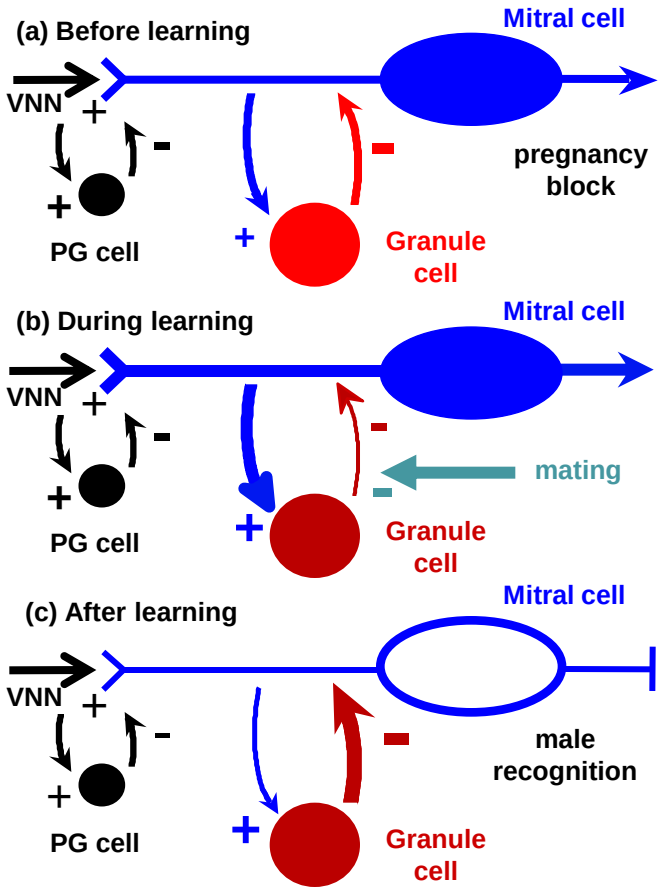
5-Figure(s)


Attractant pheromone, e.g., darcin
Non-volatile signature mixture, e.g., MUPs/MHC peptides
Volatile signature mixture
Mouse urine
AOB
MOB
Medial Amygdala
No attraction
Attraction
Attraction
Naïve - volatiles only
Exposure non-volatiles and volatiles
Experienced – volatiles only

5-Figure(s)


Noradrenaline
Idoxan (X)
Clonidine (√)
Bicuculline (√)
GABA
Excitation by mate's chemosignals
PKCα
Poly B (X)
Mitral cell
Glu
DCG-IV (√)
m2
iGluRs
DNQX (√)
APV (√)
DNQX + APV (X)
DCG-IV (√)
Ca2+
Vm
VGCC
PKC NOS
SNP(√)
Poly B (X)
Anisomycin (X)
Protein synthesis
Granule cell
= antagonist/inhibitor
= agonist/activator
(√) = promotes memory/LTP
(X) = prevents memory/LTP

5-Figure(s)


(a) Before learning
Mitral cell
VNN
+
-
pregnancy
block
+
PG cell
+
Granule
cell
-
(b) During learning
Mitral cell
VNN
+
-
mating
+
PG cell
+
Granule
cell
-
(c) After learning
Mitral cell
VNN
+
-
male
recognition
+
PG cell
+
Granule
cell
-