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Review

From sexual attraction to maternal aggression: When pheromones change their behavioural significance

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ABSTRACT

This article is part of a Special Issue "Chemosignals and Reproduction".

This paper reviews the role of chemosignals in the socio-sexual interactions of female mice, and reports two experiments testing the role of pup-derived chemosignals and the male sexual pheromone darcin in inducing and promoting maternal aggression. Female mice are attracted to urine-borne male pheromones. Volatile and non-volatile urine fractions have been proposed to contain olfactory and vomeronasal pheromones. In particular, the male-specific major urinary protein (MUP) MUP20, darcin, has been shown to be rewarding and attractive to females. Non-urinary male chemosignals, such as the lacrimal protein ESP1, promote lordosis in female mice, but its attractive properties are still to be tested. There is evidence indicating that ESP1 and MUPs are detected by vomeronasal type 2 receptors (V2R).

When a female mouse becomes pregnant, she undergoes dramatic changes in her physiology and behaviour. She builds a nest for her pups and takes care of them. Dams also defend the nest against conspecific intruders, attacking especially gonadally intact males. Maternal behaviour is dependent on a functional olfactory system, thus suggesting a role of chemosignals in the development of maternal behaviour. Our first experiment demonstrates, however, that pup chemosignals are not sufficient to induce maternal aggression in virgin females. In addition, it is known that vomeronasal stimuli are needed for maternal aggression. Since MUPs (and other molecules) are able to promote intermale aggression, in our second experiment we test if the attractive MUP darcin also promotes attacks on castrated male intruders by lactating dams. Our findings demonstrate that the same chemosignal, darcin, promotes attraction or aggression according to female reproductive state.

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Introduction

Rodents, and specifically mice (*Mus musculus domesticus*), constitute useful models to understand the neural and endocrine basis of social behaviours. Like in most other mammals, rodent social behaviours include sexual interactions (intersexual attraction and mating), agonistic encounters in which competitors fight for a territory and for mates, and parental care and other behaviours that increase the likelihood of offspring survival, e.g. nest building and pup defence. Being macrosmatic animals with highly developed olfactory and vomeronasal epithelia (plus other minor sensory organs such as the septal organ of Masera and the ganglion of Grueneberg; see Fortes-Marco et al., 2013), rodents mainly use chemosignals for intraspecies communication. Combined lesions of the main and accessory olfactory systems have devastating effects on their social interactions (see Pfeiffer and Johnston, 1994). This is because rodents emit a myriad of pheromones, e.g. chemosignals that trigger specific social behaviours and/or neuroendocrine responses in conspecifics (Karlson and Luscher, 1959).

Females of laboratory mice (and of other rodents) constitute an especially interesting model to analyse how chemosignals are processed for the control of behaviour. In contrast to males, laboratory female mice do not attack intruders to defend their territory or to get access to possible mates, but they are usually engaged in affiliative interactions with conspecifics (although interfemale aggression is present in some wild-stock mice, Stockley et al., 2013). However, for the first 10 days after parturition, dams not only show pup-directed maternal behaviour (arched back posture, lactation, pup grooming and licking), but also attack conspecifics approaching the nest. Since pup killing is pervasive among wild rodents, attacks against intruders are usually interpreted as a defence of the pups, thus being called maternal aggression (Lonstein and Gammie, 2002).

Analysis of the changes that occur in female social behaviour after parturition and their physiological substrates constitutes an interesting issue that could prove very helpful for understanding the neuroendocrinology of social interactions. A first question in this respect is whether pheromone sensing is equally involved in female-to-male reactions throughout the life of the female. Two alternative, but not necessarily exclusive possibilities exist. On the one hand, changes in female physiology (e.g. endocrine state) and/or behavioural stimulation (e.g. interaction with pups) occurring through pregnancy, parturition and lactation might alter the pattern of receptor expression in the vomeronasal and/or olfactory epithelia (e.g. Alekseyenko et al., 2006), or the responsiveness of olfactory and/or vomeronasal sensory neurons to their ligands (Stowers and Dey, 2014). A similar phenomenon has been suggested for the vomeronasal system of males in relation to their paternal vs infanticide behaviour (Tachikawa et al., 2013). As a consequence, lactating dams might detect new chemosignals derived from conspecifics or, alternatively, they might fail to detect chemosignals that they detected before. This would result in a change in social behaviour. Alternatively, females might detect the same male pheromones throughout their lives, but changes in the central nervous system during pregnancy, parturition and lactation would alter the response to these chemosignals thus modifying the social behaviour of females.

In this essay we assess these hypotheses concerning changes in the response of female mice to male chemosignals. Male-derived chemicals attract females during most of their life but, during a specific period after parturition, lactating dams fiercely attack male intruders. We first review evidence that these responses, attraction to males and attacks towards male intruders, are mediated by chemosignals detected by the olfactory and/or vomeronasal epithelia. As we will see, it seems that vomeronasal stimuli are needed for both sexual attraction and attacks on intruders, but olfactory inputs also are likely to be involved. Chemosignals promoting aggressiveness against intruders might arise from the female's pups, thus corresponding with experimental evidence that mother–pup interactions are needed to maintain maternal aggression (Gandelman, 1972). On the other hand, intruder-derived chemosignals could be the key stimuli for maternal aggression, thus explaining differential attack intensity towards different types of intruders reported in the literature (Gandelman, 1972; Rosenson and Asheroff, 1975; Bean and Wysocki, 1989).

Finally we will describe and discuss the results of two experiments that demonstrate that: a) attacks on male intruders by lactating females are promoted by a testosterone-dependent factor expressed by intruders; b) contact with the pups is necessary but not sufficient for developing nest defence; and c) the recombinant sexually attractive male pheromone darcin, when sprayed onto castrated males used as intruders, is able to promote aggression from lactating female mice.

Role of chemosignals in intersexual attraction in mice

In most animals, intersexual attraction involves emission of signals by males (songs, odours, displays) that attract females. Male mice mainly use ultrasonic vocalisations (Nyby et al., 1981; Holy and Guo, 2005) in the range of 30–110 kHz, and chemical signals that they release in their territory by means of urine marking behaviour (Hurst, 1987). Chemical cues are, however, preeminent since male mice sing ultrasonic courtship vocalisations in response to conspecific urinary odours (Guo and Holy, 2007).

In agreement with their role in intersexual attraction, male-derived chemosignals are attractive to adult but not to pre-pubertal female mice that, in fact, avoid them (Drickamer, 1989; Mucignat-Caretta et al., 1998). This can be observed using simple two-choice tests in the laboratory, in which females are left to freely explore two sources of chemosignals. Adult females prefer male- to female-derived chemosignals whether presented as an entire anaesthetised animal, a drop of urine or soiled-bedding. Similarly, adult females also prefer intact to castrated male-derived odours (Martinez-Ricos et al., 2007; DiBenedictis et al., 2012). This indicates that males produce a testosterone-dependent chemosignal that is attractive to adult females, but aversive to prepubertal females. Moncho-Bogani et al. (2002) tested this attractiveness in females that had been reared in the absence of adult male chemosignals. These so-called “chemically naïve” adult females already preferred male-soiled bedding to the more familiar female-soiled bedding even during their first experience with male chemosignals. Therefore, these chemosignals comply with the original definition of pheromones (Karlson and Luscher, 1959), as they trigger stereotyped, hardwired or non-learned responses in conspecific females.

Volatile male sexual pheromones: chemical species and their detection

The identity of the attractive male pheromone(s) has been a controversial issue. There is an open debate about the volatile and/or non-volatile nature of these male sexual pheromones, as well as about the sensory organ responsible for their detection, namely the olfactory epithelium or the vomeronasal organ. Consequently, it is also unclear what kind of olfactory or vomeronasal receptors can detect the attractive male-derived chemosignals. As discussed by Fortes-Marco et al. (2013), these hardwired responses require highly specific receptors. This excludes the canonical, generalist olfactory receptors. Instead,

pheromone detection might depend on specific trace amine-associated receptors or guanylyl cyclase receptors in the olfactory epithelium (and other sensory organs) and/or type 1 (V1R) or type 2 vomeronasal receptors (V2R) expressed by cells in the apical and basal halves of the vomeronasal epithelium, respectively.

The search for sexual pheromones in mice started with looking for male-specific urinary volatiles. This led to the identification of four compounds, α- and β-farnesenes, dehydro-exo-brevicomin (brevicomin) and 2-(sec-butyl)-4,5-dihydrothiazole (thiazoline) that are present in the urine of intact adult males but not of immature and castrated ones (Harvey et al., 1989). Whilst farnesenes are preputial secretions that are present in voided but not in bladder urine, thiazoline and brevicomin are urinary compounds. Jemiolo and collaborators tested the attractive properties of these compounds for adult females. They showed (Jemiolo et al., 1985) that adding a mixture of brevicomin and thiazoline to the urine of castrated males at concentrations simulating the amount of these volatiles in normal male urine, made this urine as attractive as urine of gonadally intact males to virgin females. Farnesenes induce preferential chemoinvestigation by virgin female only if they are spiked at very high (supra-physiological) concentrations (250/500 ppm) in bladder urine or water (Jemiolo et al., 1991). By contrast, sexually experienced females recognize and prefer samples with lower concentrations of farnesenes (10 ppm, twice the level in voided urine), thus suggesting that farnesenes are odorants that become attractive by their association with sex or other innately attractive chemosignals. Therefore, farnesenes do not fulfil the definition of a pheromone.

These volatiles can be olfactory and/or vomeronasal stimuli. In vitro electrophysiological studies (Leinders-Zufall et al., 2000) indicate that farnesenes, brevicomin and thiazoline (among other chemical cues for which a pheromonal effect has been proposed) are detected at very low concentrations ($<10^{-10}$ M) and with a high specificity by vomeronasal cells probably expressing V1R (given their apical location in the VNO epithelium). More recent studies indicate that other urine-borne volatiles secreted by the preputial glands of males (Zhang et al., 2008), namely hexadecanol and hexadecyl acetate, are also vomeronasal stimuli that elicit female attraction. All these data strongly suggest a role of volatile vomeronasal stimuli in intra-species communication, including intersexual attraction.

In addition, urine-borne male-derived volatiles can also be odorants, e.g. olfactory rather than vomeronasal stimuli, with attractive properties for females. Thus, farnesenes and other volatile vomeronasal ligands stimulate the olfactory epithelium (Wang et al., 2006). Lin et al. (2005) combined gas chromatography with simultaneous recording of the activity of mitral cells in the main olfactory bulb stimulated by individual volatiles in mouse urine. Using this approach, they realised that a subpopulation of the mitral cells that responded to urinary volatiles were activated by a specific odorant present only in male (20 ppb), but not in female or castrated male urine. The elution time of this compound in the gas chromatography column corresponded to (methylthio)methanethiol (MTMT). Adding 20 ppb of synthetic MTMT to urine from castrated males made it attractive to sexually experienced females when compared with non-supplemented castrated male urine. However, females showed no preference for 20 ppb in water and only slight preference for water containing 50 ppb MTMT versus pure water. Other findings suggestive of an olfactory nature of urine-borne male sexual attractants derive from research by the group of Michael Baum and Julie Bakker (Keller et al., 2006a, 2006b; see Baum, 2012), who showed that female preference for intact vs castrated male-derived volatiles was abolished by lesions of the olfactory epithelium but preserved after lesions of the vomeronasal organ.

However, in most of these studies the chemical and even the sexual experience of the subject females undergoing preference tests were not properly controlled. This is a key point in the identification of pheromones, since previous experience allows learning processes that cast doubts on the innateness and consistency of the attraction observed and, consequently, on the pheromonal nature of the volatiles being tested. As we will see this is a controversial, open question. The importance of chemical experience was underlined by the experiments with chemically naïve outbred female mice (CD1 or ICR) by Moncho-Bogani et al. (2002). Their results showed that innate preference for male- over female-soiled bedding was not observed if contact with the bedding was prevented during the test by means of a perforated platform that allowed access of the animals only to volatile compounds. This contrasts with the previously discussed data, as it indicates that the primary attractive male pheromone(s) is not airborne, but it has, at least, a fundamental component detected only on contact. Moncho-Bogani et al. (2002) also showed that if the females were allowed to freely chemoinvestigate (contact allowed) male-soiled bedding for 15 min in four consecutive days, they became attracted by airborne male-derived volatiles. This was interpreted as a case of classical conditioning in which an unconditioned attractor, the non-volatile pheromone, was associated with the volatiles emanating from male-soiled bedding that, consequently, elicited a conditioned attraction. Ramm et al. (2008) replicated these results by using wild-derived female mice (F2–F4 generations), and showed that the odour signature of a specific male becomes attractive by its association with a non-volatile sexual pheromone. According to these findings, the preference of the females for male-derived volatiles observed in many previous works (see above) could be explained as resulting from early experience with adult male chemosignals (e.g. during peri-pubertal life, prior to the arrival of females in the laboratory as adults). This possibility was recently tested by Jouhanneau et al. (2014), who checked the impact of peripubertal experience with male-soiled bedding on adult preference for volatiles. Their results indicate that even animals having no experience with any kind of male chemosignals during pre or peripubertal life, show attraction for male-derived volatiles in a Y maze preference test, thus reinforcing the view that attraction to volatiles is innate.

These conflicting results on the innate versus learned attractiveness of male-derived volatiles to females might be related to several variables. First, experiments showing innate attraction of females for male-derived volatiles use inbred strains, whereas those rendering negative results (no innate attraction for volatiles) use either outbred strains or F2–F4 descendants from wild-derived mice, thus suggesting inter-strain differences. It is interesting to note that some peptidic chemosignals are not expressed in some lab mouse strains (e.g. Kimoto et al., 2007; Roberts et al., 2010). Further, within inbred strains, female odours used as control scents come from donors genetically identical to subject females and thus are highly familiar, whilst intact male odours differ substantially from scents that the female has encountered previously despite coming from the same strain. This difference in familiarity may stimulate greater investigation of male scent because of its novelty. However, female odours normally differ between individuals among outbred and wild-stock mice, so in this case both female and male test odours will be unfamiliar to the subject. A second important variable that might explain the conflicting results reported in the literature might be related to the test conditions resulting in different accessibility to the stimuli. In many instances, preference tests performed in Y-mazes with a fan-generated airflow tend to render positive results, whereas most negative results derive from tests in which animals must track the source of the chemosignals by exploring an arena with no airflow transporting the volatiles. MUPs are small proteins that attach to dust particles and readily become airborne in an airflow. In fact, MUPs are the main aeroallergen in animal facilities, responsible for the common allergic response of humans to mice or rats (e.g. Gordon et al., 1997). Therefore, it is possible that the use of an airflow system could expose animals to involatile as well as volatile scent components, including MUPs.

However, the discrepant results could also be a consequence of the modulatory role of sexual steroids on the preference for male-derived volatiles in females. For instance, the negative results by Moncho-Bogani et al. (2002) were obtained in females in which the oestrous

cycle was not controlled. It is likely that, when tested for the first time for male odorant attraction, most of the females were in diestrus/metestrus, which together constitute ≈75% of the oestrous cycle (Byers et al., 2012). By contrast, in most experiments by Keller and coworkers that found innate attraction for male volatiles, female mice were treated with steroids (estradiol implants plus progesterone injections) to induce a behavioural oestrus, thus suggesting that preference for male urinary volatiles might be restricted to oestrus. If this explanation was correct, the innate attraction for male volatiles would differ from that for non-volatile pheromones, which is present in ovariectomized females (Moncho-Bogani et al., 2004) even though there is some evidence for steroid modulation of VNO sensitivity (see Baum and Bakker, 2013).

Nevertheless, several lines of evidence contradict this explanation. First, Jemiolo et al. (1985) tested this idea and concluded that: "female's attraction to male odours was equally strong on days that they displayed vaginal oestrus to days of anoestrus". In addition, recent studies by Roberts et al. (2010) using wild-derived female mice revealed a lack of preference for male volatiles, even if females in proestrous/estrous were employed. Therefore, the innate attraction of female mice towards male derived volatiles under some circumstances is still an open issue that requires further investigation.

Non-volatile male sexual pheromones: attraction and promotion of lordosis in females

According to Wysocki et al. (1980), the vomeronasal organ (VNO), but not the olfactory epithelium (with few exceptions, see Spehr et al., 2006), is able to detect non-volatile chemicals in the environment. Therefore, irrespective of the existence of volatile sexually attractive male pheromones, non-volatile male pheromones are detected by the VNO of females in which they elicit attraction and other behavioural responses. In fact, VNO lesions in female mice (Keller et al., 2006b) suppress their preference to sniff-touch the body, urine or soiled-bedding of male mice, as compared to the same stimuli from females or castrated males. In the same vein, lesions of the accessory olfactory bulb (AOB) suppress preference for male-soiled bedding in chemically naïve females (Martinez-Ricos et al., 2008), even if these females are able to detect and discriminate male urine from other odorants.

At least two non-volatile male-specific pheromones have been identified to date that are involved in the control of socio-sexual behaviour, the peptide ESP1 and major urinary proteins (MUPs). When detected by vomeronasal organ, ESP1 stimulates lordosis in females (Haga et al., 2010), whereas some MUPs have been shown to elicit aggression and countermarking in males (Chamero et al., 2007, 2011; Kaur et al., 2014) and attraction in females (Roberts et al., 2010). In addition, type I MHC peptides, showing low volatility, may also act as chemosignals that allow mate recognition in the context of the induction of pregnancy failure (Bruce effect; Leinders-Zufall et al., 2004).

The lacrimal gland of pubertal and adult male mice (from 4 weeks of age) secretes a 7 kDa peptide known as ESP1 (Exocrine Secreted Peptide 1), which specifically stimulates VNO cells expressing V2Rp5 vomeronasal receptor (Kimoto et al., 2005; Haga et al., 2010). These vomeronasal cells co-express non-classical class I major histocompatibility genes, also called *H2-Mv* genes, which apparently contribute to their ultrasensitivity to ESP1 (Leinders-Zufall et al., 2014). In fact, using electrovomerograms Leinders-Zufall et al. (2014) determined that the EC_{50} for ESP1 was in the picomolar range in vitro. Accordingly, when applied into the oronasal grove of adult females, 1 μM ESP1 induces robust c-fos activity in mitral cells of the caudal AOB (Leinders-Zufall et al., 2014).

The presence of a lacrimal protein acting as a pheromone fits previous observations by Luo et al. (2003), who recorded the electrophysiological activity of mitral cells of the accessory olfactory bulb in freely moving mice, whilst they were investigating several stimuli. Some mitral cells of the AOB were specifically activated when the subject sniffed-touched the face and snout of an anaesthetised conspecific. In this respect, it is interesting to note that, although ESP1 is exclusively produced by the lacrimal gland, the harderian, lacrimal and submaxillary glands produce other peptides of the ESP family that are not expressed in a sexually dimorphic way. At least some of these peptides apparently activate VNO cells (Haga et al., 2010), thus suggesting a role of the ESP peptides in social signalling. For instance, it has recently been described that ESP22 is secreted by lacrimal glands of both male and female mice, in a 50-fold higher concentration in juvenile than adults (Ferrero et al., 2013). ESP22 apparently acts as a juvenile pheromone that inhibits sexual behaviour in adults. Like other ESP peptides, ESP22 is detected by VNO cells, likely expressing V2R.

However, urine is usually considered the most important source of chemosignals in mice, which fits the conspicuous behaviour of urine marking and countermarking exhibited by male mice (see Kaur et al., 2014). To date, an attractive non-volatile pheromone has been identified in the urine of male mice, which has been named darcin (Roberts et al., 2010). It is a member of the major urinary protein family, MUP20 (MGI: 3651981), which shows a strongly male-biased expression and a molecular weight of 18,893 Da. Being a lipocalin, darcin binds small lipophilic molecules, with high affinity for thiazoline (Armstrong et al., 2005) which, as discussed above, is a volatile compound also present in male (but not female) urine. Although some pheromonal activities have been proposed for thiazoline, including attraction (together with brevicomin, but not alone), oestrous synchronisation and puberty acceleration (Jemiolo et al., 1985, 1989; Novotny et al., 1999), its role in intersexual attraction must be negligible since recombinant darcin obtained in *Escherichia coli*, free of ligands, is attractive when compared to buffer or to full female urine. In fact, another MUP isoform that also binds thiazoline (MUP 18,694 Da) shows no attractive properties (Roberts et al., 2010), thus demonstrating that darcin has actual pheromonal properties, rather than being a simple odorant or pheromone reservoir.

The attraction that female mice display for male sexual pheromones is a reflection of the reinforcing properties of these pheromones for females, and the learning that follows the acquisition of rewarding stimuli. This was first shown by Martinez-Ricos et al. (2007), who tested the ability of bedding soiled by gonadally intact males, females or castrated males to induce place preference. To do so, adult, chemically naïve females were run in successive preference tests (one test per day, four consecutive days) of soiled vs clean bedding, with the soiled bedding present in the same location in the cage in every test. On the next day, a clean vs clean bedding preference test revealed preference for the location of the cage where soiled bedding had been presented in previous tests, but only among those females that were presented with soiled bedding from intact males. By contrast, bedding soiled by castrated males or females did not generate a place preference in females. In addition, if a platform separated the females from the bedding, leaving access only to the volatiles it delivers, neither a preference during the four test days nor a place preference was observed. Therefore, some non-volatile chemosignals contained in the bedding soiled by males (but not castrated males or females) are both attractive and rewarding/reinforcing (able to generate place preference) for females. More recently, Roberts et al. (2012) replicated and expanded these findings by using urine, rather than soiled bedding, as a source of pheromones. Moreover, they demonstrated that darcin, the attractive male sexual pheromone, was the key element that makes male urine rewarding for females: recombinant darcin induces place preference. Interestingly, darcin induces attraction and spatial learning not only in females but also in males that, nevertheless, are less attracted to their own than to a competitor's urine. In addition, they also demonstrated that urine volatiles that had become secondary attractors by means of their association with darcin (once again, volatiles were found not to be not primarily attractive) were not able to induce place preference. All these findings strongly suggest that darcin is the main male sexual pheromone of mice, due to its rewarding properties for females, which

mediate an interesting type of spatial learning especially adaptive for mate search (see discussion in Roberts et al., 2012).

It is worth noting that the two identified non-volatile peptidic pheromones involved in sexual attraction (darcin) and mating (ESP1), although expressed at high levels in wild house mice (*M. musculus domesticus*) are not expressed in some strains of laboratory mice. For instance, BALB/c male mice show very low (virtually undetectable) levels of darcin in their urine (Roberts et al., 2010), whereas ESP1 is absent in the lacrimal fluid of the males of most of the lab strains with the exception of BALB/c and DBA (Haga et al., 2010). This important inter-strain heterogeneity might also affect the composition of the excreted/secreted volatiles. As discussed above, this might explain some of the contradictory results found in the literature concerning the role of volatiles in innate intersexual attraction in mice.

Role of chemosignals in aggressive behaviour: hints on maternal aggression

Analysis of the role played by chemosignals in aggression is more difficult than their role in attraction. Except for very special experimental situations (e.g. optogenetic stimulation of the aggression hypothalamic centre, Lin et al., 2011) aggression is highly context-dependent. An animal seldom attacks immobile objects, but only conspecifics moving in the owner's territory. For this reason, the standard test for analysing the neurobiology of aggression is the resident–intruder paradigm. Thus, for intermale aggression, a male (intruder) is introduced in the home cage of another (resident). The resident male reacts, sooner or later, attacking the intruder, and the latency, number of events and time attacking can be easily measured (Scott, 1966).

The use of this experimental paradigm indicates clearly that, in the context of intermale aggression, chemosignals are critical in mice. Castrated male intruders are attacked only if sprayed with urine from a gonadally intact male (Mugford and Nowell, 1970). This indicates that some urine-borne molecules are signalling the maleness of the intruder and consequently eliciting aggression from the resident. This strategy allows analysis of both the organs involved in the detection of male aggression-promoting signals, and the nature and identity of those chemosignals. For the first aim, one should study the effect of lesions or genetic manipulations of the sensory organs of the resident mouse on attacks towards the intruder. For the second, intruder castrated males can be swabbed with solutions of specific compounds or urine fractions, to check their role as aggression promoting pheromones. As we will see, data on maternal aggression are scarcer than those on male–male aggression but suggest a similar, fundamental role of chemical signals.

Role of the vomeronasal and olfactory epithelia on aggression

Ablation of the vomeronasal organ in males (Clancy et al., 1984) and females (Bean and Wysocki, 1989) impairs intermale aggression and maternal aggression respectively. The effects of these lesions seem largely independent of previous sexual or chemosensory experience, thus indicating that aggression-promoting pheromones are detected by the VNO in both males and lactating females. This is strongly reinforced by analysis of the social behaviour of mice with transgenic modifications of genes involved in vomeronasal sensory transduction.

Thus, null mutants for trpc2, which encodes the transient receptor potential channel responsible for most of the vomeronasal sensory transduction (but see below), show no aggression. Male trpc2$^{-/-}$ mutants do not attack gonadally intact males (Leypold et al., 2002) or castrated males swabbed with urine of intact males (Stowers et al., 2002), but try to mount them instead, as if they interpret these intruders as females. When lactating females are used in maternal aggression tests, nest defence is also clearly impaired in trpc2$^{-/-}$ mutants (Leypold et al., 2002; Kimchi et al., 2007) even when the mutation is shifted into a line of mice selected for their robust maternal aggression (Hasen and Gammie, 2009, 2011). This indicates that vomeronasal stimuli elicit both intermale aggression and maternal aggression. However, data on trpc2$^{-/-}$ mice should be interpreted with caution since they seem to retain some type of vomeronasal chemodetection (Kelliher et al., 2006), probably due to the existence of alternative signalling cascades that depend on other channel currents (Yang and Delay, 2010; Kim et al., 2011, 2012). In addition, trpc2$^{-/-}$ mutants probably have altered olfactory sensitivity (Omura and Mombaerts, 2014). In spite of these caveats, these findings suggest that maternal aggression and intermale aggression similarly depend on vomeronasal chemosignals, detected by V1R, V2R or both.

This is further supported by behavioural analysis of specific mutants for the G proteins associated to these receptors, namely Gi2 for V1R and Go for V2R (see Fortes-Marco et al., 2013), which differ in their α subunit. In relation to V2R-mediated chemoreception, Gαo mutants (Chamero et al., 2011) show reduced intermale aggression and maternal aggression. In addition, Loconto et al. (2003) analysed a mouse mutant line knockout for β2-microglobulin (generated by Zijlstra et al., 1990), a protein expressed in vomeronasal neurons where it contributes to escort V2R to the cell surface. Consequently, in these mutants V2R are misallocated and pheromone detection through V2R is expected to be deficient. In agreement with the results of Gαo knockout mice, β2-microglobulin knockout male mice show no aggression, although in contrast to trpc2$^{-/-}$ mutants, they do not mount male intruders. To our knowledge no studies on maternal aggression have been published for these mutants. A third mutant showing reduced V2R sensitivity renders results consistent with the idea that these receptors are involved in detecting chemosignals that promote aggression. Thus, as discussed above, a subpopulation of the basal, V2R-expressing cells of the vomeronasal epithelium co-express nine nonclassical class I major histocompatibility complex (MHC) genes, termed H2-Mv (Ishii et al., 2003). Homozygous mice lacking these genes (ΔH2-Mv mice) produced by chromosome engineering, not only show reduced response to several V2R ligands but also fail to display intermale aggression and maternal aggression in the presence of male urine-swabbed castrated male intruders (Leinders-Zufall et al., 2014). These data indicate that this subpopulation of V2R-expressing cells is also critical for the expression of aggression by males and females. Whether the rest of the basal cells of the vomeronasal epithelium also play a role in the detection of aggression-promoting chemosignals is unknown.

Concerning V1R, knockouts for the Gαi2 (Norlin et al., 2003) show defective intermale aggression and maternal aggression. This suggests that, simultaneous signalling through both V1R and V2R is required for the expression of aggression. When one or the other vomeronasal subsystems fail, no aggression is expressed (Chamero et al., 2011). An exception was revealed by Del Punta et al. (2002), who used chromosome-engineering technology to generate a mouse line with a deletion of a cluster of 16 genes that code for V1R. This mutation abolishes maternal aggression but not intermale aggression. Therefore, within the V1R vomeronasal subsystem, it seems that whereas maternal aggression depends on some of these 16 V1R, intermale aggression depends on the remaining V1R. Some yet unknown chemosignals detected by one or several of these 16 V1R receptors, are critical for the expression of maternal aggression but do not promote intermale aggression.

The role of olfactory stimuli (airborne odours) in maternal aggression is less clear and the results of lesions or mutations of the main olfactory system are difficult to interpret. Lesions of the olfactory epithelium using $ZnSO_4$ infusion into the nasal cavity and/or lesion of the main olfactory bulbs have mild or severe effects (respectively) on nest building and maternal care (Vandenbergh, 1973), but the effects on maternal aggression have not been properly tested, mainly because of death of the pups due to maternal neglect or cannibalism (see below). On the other hand, null mutations for type 3 adenylyl cyclase (AC3$^{-/-}$; Wang and Storm, 2011), an enzyme involved in sensory transduction in olfactory neurons have been used to study the role of olfaction in maternal

behaviour including aggression. These AC3$^{-/-}$ females are not only anosmic (as expected), but also do not build a nest or retrieve pups. Interestingly, these behavioural deficits are observed in both lactating dams and virgin female mutants (wild type laboratory virgin female mice show spontaneous pup care, Stolzenberg and Rissman, 2011; our unpublished observations).

Importantly, AC3$^{-/-}$ dams do not show maternal aggression (Wang and Storm, 2011). Although this may be attributed to the lack of sensitivity of these mutants to specific pup- and/or intruder-derived aggression-promoting odorants, there are alternative explanations for this behavioural deficit. On the one hand, since anosmic females do not build a nest or gather pups into it, maybe their lack of aggression towards intruders simply reflects this deficit, e.g. there is no maternal motivation and/or there is no nest to defend. On the other hand, the lack of odour sensitivity might also have an indirect effect on VNO-driven maternal aggression. Thus, as Mandiyan et al. (2005) suggest, one of the main effects of odorants is to promote VNO-mediated chemoinvestigation requiring contact with the substrate and vomeronasal pumping. Therefore, anosmic mutants (in this case cyclic nucleotide-gated channel α2) do not sniff at conspecifics or their urine and, consequently, they may not have access to vomeronasal stimuli, in spite of their having a functional VNO.

Interestingly, female mice (Gandelman et al., 1971b) and rats (Fleming and Rosenblatt, 1974) undergoing complete olfactory bulb lesions show not only a lack of maternal care but also a high level of cannibalism of their own pups. Pup killing is also frequent in primiparous female mice in which the olfactory epithelium is lesioned with $ZnSO_4$ intranasal irrigation (Seegal and Denenberg, 1974). An interesting possible explanation for this unexpected effect of olfactory deprivation would be that olfactory cues from the pups protect them from maternal aggression, in a way similar to that reported for chick vocalisations in turkeys by Schleidt et al. (1960). When a female turkey is deafened she is able to become pregnant, lay eggs and incubate them normally. However, as soon as chicks hatch, the mother pecks them to death. As the authors concluded and Lorenz (1963) discussed in detail, these data suggest that turkey hens show non-specific maternal aggression towards any unfamiliar object approaching the nest, but the chicks' cheep inhibits maternal aggression. This hypothesis was tested and proven by presenting a stuffed chicken to a brooding turkey with normal hearing. The stuffed chick was attacked unless a built-in loudspeaker emitted the typical call of a turkey poult. In the same way, data on olfactory deprivation in mice suggest that some as yet unidentified olfactory cues protect pups from being attacked by their mothers. This view is supported by the fact that pup cannibalism follows the same evolution as maternal aggression, reaching a maximum immediately after parturition and declining thereafter to disappear about two weeks postpartum (Gandelman et al., 1971a, 1972). However, there are no reports of pup killing when anosmia is induced by a mutational deficit (Cnagα2$^{-/y}$, Mandiyan et al., 2005; AC3$^{-/-}$, Wang and Storm, 2011).

An alternative explanation could be that non-olfactory effects of the lesion approach, such as stress, might induce pup killing. For instance, olfactory bulb lesion in rodents has been employed as a model for depression in both rats (Song and Leonard, 2005) and mice (Sato et al., 2010). The behavioural, physiological and neurochemical alterations induced by bulbectomy are similar to those observed in clinical patients with depression symptoms. In addition, bulbectomy has a deep impact on maternal behaviour. For instance, bulbectomised dams spent less time licking/grooming and crouching their pups, and increased total time out of nest (Sato et al., 2010). The authors noted that these deficits reduced pup survival, but they do not mention cannibalistic behaviour. Although these data suggest that pup-killing might be secondary to depression, it does not fit the induction of cannibalism by peripheral anosmia induced by nasal irrigation with $ZnSO_4$ (Seegal and Denenberg, 1974), which has not been related to depression.

As a conclusion, maternal behaviour seems to be dependent on an interplay between olfactory and vomeronasal cues. On the one hand, pup-derived olfactory cues allow the development of maternal behaviour and may also protect pups from maternal aggression. On the other hand, vomeronasal chemosignals promote aggression towards intruders. We will now review data on the nature and identity of these chemosignals.

Chemosignals promoting aggression

Lactating female mice attack gonadectomised intruders less than gonadally intact ones, whether males or females (Gandelman, 1972; Rosenson and Asheroff, 1975). These data suggest that at least some of the male and female chemosignals that promote maternal aggression are produced in a way dependent on sexual steroids. Since most of the studies on aggression are based on intermale aggression, where the intruder is by definition a male, attempts to identify aggression-promoting chemosignals have focused on testosterone-dependent urine-borne compounds. Similar to the identification of male attractive pheromones, there is an open debate on the exclusive or complementary role of volatile and non-volatile chemosignals in stimulating aggression.

In the 1980's, Novotny et al. (1985) demonstrated a role for male-derived urinary volatiles in intermale aggression. They showed that resident males attacked a castrated male intruder when it was swabbed with urine from castrated males supplemented with both brevicomin and thiazoline at their natural concentrations in male urine. The attack latency and duration towards these castrated males were similar to that shown towards castrated males sprayed with urine from intact males. Neither of these volatiles was active alone, however. In addition, an aqueous solution of the mixture of both volatiles was not effective in promoting aggression against castrated males. These data led the authors to conclude that both compounds act synergistically with some unknown elements present in the urine of castrated males to promote intermale aggression. As discussed above, this mixture was shown to have attractive properties for females (Jemiolo et al., 1985). Although the low molecular weight fraction of male urine also seems to promote aggression by lactating females towards castrated intruders approaching the nest (see below and Chamero et al., 2011), there is no direct evidence that brevicomin and/or thiazoline are involved in maternal aggression.

More recently, Chamero et al. (2007) analysed the presence of intermale aggression-promoting compounds in the urine of male mice. To do so, they first fractioned urine into a low molecular weight fraction (LMW) containing most free volatiles, and a high molecular weight fraction (HMW) composed mainly of MUPs and their accompanying volatile ligands. Then, they tested the aggression-promoting properties of these fractions by spraying them onto castrated male intruders and analysed the reaction of male residents. Their results indicate that the LMW urine fraction promotes aggression against castrated male intruders. Although LMW fraction might also contain some compounds with relatively low volatility (e.g. MHC type I peptides), this suggests that volatile urine compounds, likely detected by V1R vomeronasal receptors (Leinders-Zufall et al., 2000), are able to elicit aggression. In fact, knockout mutant mice for the Gαi2 protein (Norlin et al., 2003), which is a key element in the transduction cascade of V1R expressing vomeronasal neurons, are deficient in intermale aggression. Although, to the best of our knowledge, there are no studies on the possible role of volatile male urine compounds in promoting maternal aggression, it is interesting to note that lactating females defective for V1R signalling (Del Punta et al., 2002; Norlin et al., 2003) do not attack male intruders. Therefore, V1R-detected chemosignals are involved not only in intermale aggression but also in nest defence. However, the identity of these presumably volatile chemosignals promoting maternal aggression remains unknown.

In addition, Chamero et al. (2007) observed that the HMW urine fraction also elicits male aggression towards a castrated male intruder. The authors further treated this urine fraction to demonstrate that

MUPs are the main (if not the only) component of this HMW fraction that elicits attacks on castrated intruders. First, protease treatment suppressed the aggression-promoting properties of this urine fraction. Second, menadione-treated HMW (in which many but probably not all volatile ligands are displaced from MUPs) retained aggression-promoting activity. And third, a mixture of four of the MUPs present in the urine of C57BL/6J male mice, produced in *E. coli* by recombinant technology, was able to induce full aggressive behaviour towards castrated male intruders. A recent study (Kaur et al., 2014) has identified two MUPs eliciting intermale aggression, e.g. Mup3 and Mup20.

In vitro calcium imaging and electrophysiological recording of VNO cells have shown that MUPs activate basal (Kaur et al., 2014), Gαo-expressing cells (Chamero et al., 2007), very likely through V2R. For this activation to occur, β2-microglobulin is needed, thus fully supporting previous findings by Loconto et al. (2003) on the role of V2R/β2-microglobulin in intermale aggression. Therefore, MUPs detected by V2R-expressing vomeronasal neurons seem to act as intermale aggression-triggering cues. This is fully consistent with the behavioural deficits displayed by mice bearing a conditional null mutation of Gαo in vomeronasal neurons ($cG\alpha o^{-/-}$; Chamero et al., 2011). Mutant $cG\alpha o^{-/-}$ male mice show a 15-fold reduction in the duration of attacks to male intruders when compared to wild-type male residents. In addition, when a resident $cG\alpha o^{-/-}$ male was used, castrated male intruders swabbed with intact male urine, its LMW fraction or the mixture of recombinant MUPs, received very few attacks. Interestingly, similar results were found for maternal aggression. Thus, whereas wild-type lactating females exhibit aggression towards castrated males sprayed with LMW male urine fraction or with a mixture of recombinant MUPs, $cG\alpha o^{-/-}$ females fail to exhibit maternal aggression (but not maternal care).

As a conclusion, V1R- and V2R-expressing vomeronasal neurons detect volatile and non-volatile male urine compounds, respectively, both eliciting aggression in male and female mice. The available data indicate that intermale aggression is mediated by the non-volatile, V2R detected major urinary proteins Mup3 and Mup20 (Kaur et al., 2014), but the volatile cues that promote aggression (detected through V1R) have not been identified yet. Surprisingly, whereas in wild-type mice one of these chemosignals alone (the LMW urine fraction, or recombinant MUPs) is able to elicit aggression against castrated males (Chamero et al., 2007), lack of function in one of the subsystems of the VNO (e.g. $G\alpha i2^{-/-}$ mutants, Norlin et al., 2003; $cG\alpha o^{-/-}$ mutants, Chamero et al., 2011) virtually abolishes intermale aggression and maternal aggression. The reason for these apparently contradictory results is still unknown (for further discussion, see Chamero et al., 2011).

Role of darcin in maternal aggression

The evidence reviewed above leads to three main conclusions. First, in mice (though probably this can be generalized to other rodents) all kinds of socio-sexual interactions are heavily dependent on chemical signals. These include intersexual attraction (attraction of females by male chemosignals is better characterized than the reverse), agonistic behaviours (intermale and maternal aggression) and maternal care. Second, the olfactory system has a fundamental role in the development of maternal care of pups, but the existence of olfactory pheromones that innately trigger sexual attraction or agonistic responses remains unclear (see Baum, 2012, for an interesting discussion), due to the bewildering effects of variables such as: a) previous chemosensory (or sexual) experience (see Martinez-Garcia et al., 2009); b) endocrine regulation (Baum and Bakker, 2013); c) possible interstrain differences; d) dual action of some volatiles through the olfactory and vomeronasal epithelia (see Fortes-Marco et al., 2013); and e) possible roles of olfactory stimuli in triggering contact-mediated vomeronasal chemoinvestigation (Mandiyan et al., 2005). And third, by contrast, there seems to be consensus that some vomeronasal stimuli trigger both intersexual attraction and aggression. Specifically, there is increasing evidence that non-volatile urinary compounds, e.g. major urinary proteins, are involved in both processes in mice.

This leads us again to the interesting issue of whether the same maleness chemosignals are responsible for attraction of females and for promoting attacks on male intruders during the postpartum period. A second interesting question is what causes females to change their behavioural response to male chemosignals, from attraction and mating facilitation to aggression after parturition (in fact a few days before, Mann et al., 1984; Bosch, 2013). In this respect, since olfactory cues (likely derived from pups) are critical for other aspects of maternal behaviour, we wondered whether intimate contact with pups providing full access to these olfactory cues might be a causal factor facilitating this change in behaviour.

In order to explore these ideas we designed and performed two experiments. In Experiment 1, we compared the aggressive response towards intact or castrated male intruders by three kinds of females: lactating dams, virgin females and "godmothers". As a negative control, we employed virgin females that had never contacted pups or youngsters, in which no aggression was expected. As a positive control we used lactating dams, which were expected to attack intruders. The third group, the "godmothers", were virgin females that had been in close contact with pups since they were born, and shared pup care with the mother (in fact they were aunts of the pups). Consequently they fulfil three important characteristics: they were fully maternal at the moment of being tested (unpublished results), they were exposed to pup stimuli (chemosensory, but also visual and auditory) for the same time as the dam, and they had a nest to defend in their home cage where they were tested for maternal aggression. This is very important, since maternal aggression is strictly context-dependent, e.g. it does not occur away from the dam's home cage containing the nest (unpublished results). *Godmothers* constitute an excellent model to test whether pup stimuli are able to induce aggressiveness in females that are already maternal or, alternatively, physiological changes related to gestation, parturition and lactation are essential. In addition, this experiment tests if, in our experimental conditions using outbred CD1 (ICR) mice, maleness chemosignals promote maternal aggression (e.g. comparing dam attacks on castrated versus intact males). The overall MUP pattern of CD1 (ICR) mice is similar to inbred strains of the Swiss and Castle lineages (Cheetham et al., 2009). Although males from some strains in these lineages do not express darcin (Cheetham et al., 2009), CD1 (ICR) males express darcin at approximately 8% of total urinary MUP content (Davidson, Roberts, Hurst and Beynon, unpublished data).

Since, as we will see, *godmothers* showed no aggression towards intruders, we decided to use them as perfect controls in a second experiment, since they differ from the dams only in the fact that they are not mothers. Thus, in Experiment 2 we tested the aggression-promoting properties of the attractive male pheromone darcin in lactating dams, using *godmothers* as non-aggressive controls. To do so, we compared the attacks of both kinds of females towards three groups of castrated males swabbed, respectively, with saline solution (vehicle), recombinant darcin (r-darcin; Roberts et al., 2010) at natural concentration, and full urine of gonadally intact males.

For these experiments, 47 adult female and 78 adult male mice (9–14 weeks of age at the beginning of the experiments) of CD1–ICR strain (Charles River Laboratories, France; Janvier Labs, France) were used. All animals were housed in polypropylene plastic cages with ad libitum access to water and food on a 12:12 light/dark cycle with lights on at 08:00 h. Cages were cleaned weekly, except during postpartum days, when dams were left undisturbed until the end of the experiment. Animals were treated throughout according to the European Communities Council Directive of 24th November 1986 86/609/ECC, and procedures were approved by the Committee of Ethics on Animal Experimentation of the University of Valencia.

Experiment 1: effect of contact with pups on nest defence against male intruders

Adult, virgin females were randomly assigned to three experimental groups: dams (n = 9), *godmothers* (n = 9) and virgin females (n = 9). Dams and stud males were group housed for 4 days (3 females/male) and following impregnation, each female was housed with a virgin female (a *godmother* female) that was a sister of the dam, in a black propylene plastic cage (145 mm wide, 465 mm long, and 215 mm high), where it remained with the dam during the rest of gestation, parturition and lactation. The day of birth was considered as postpartum day 0 (PPD 0). On PPD 2, litters were culled to 8 pups. In parallel, virgin females were housed in pairs and used as negative control animals that were devoid of contact with pups.

Intruder males, 15 castrated males and 15 intact males, were group housed (7–8 castrated animals per cage; 4–5 intact animals per cage taking care that no aggression occurred). For gonadectomy, adult males were anaesthetised with i.p. injections of ketamine (75 mg/kg; Imalgène 500, Merial, Toulouse, France) and medetomidine (1 mg/kg; Domtor, Esteve, Madrid, Spain). They also received a subcutaneous injection of butorphanol (5 mg/kg, Torbugesic, Pfizer, Alcobendas, Spain). Eye drops were applied to prevent eye ulceration during surgery. After surgery, atipamezole hydrochloride was administered (1 mg/kg i.p.; Antisedan, Esteve, Madrid, Spain) to reverse the effect of the anaesthesia. Castrated males were used for the maternal aggression tests at least three weeks after gonadectomy.

Each female was confronted with a castrated and an intact male on two consecutive days, with the order of tests counterbalanced for each group of females. In order to reduce possible damage to the males and to avoid possible effects of their experience on subsequent tests, each male was confronted with only one lactating dam and was used in no more than two maternal aggression tests in total.

Maternal aggression tests were performed in the females' home cages between PPD 3–5, when the highest level of maternal aggression occurs (Gandelman, 1972; Lonstein and Gammie, 2002), between 0900–1400 and 1500–1700 h. Female subjects were brought to the testing room in their home cage and pups were removed prior to the tests (Svare et al., 1981; Lonstein and Gammie, 2002), to avoid any potential infanticide behaviour by the intruder (Vom Saal and Howard, 1982). During the tests, pups were left in another cage close to their home cage. Since females were house in pairs (dam/*godmother*; two virgin females), the female that was not going to be tested was removed and put with the pups in the adjacent cage. This ensured proper pup care throughout the experiment. For the aggression test, an unrelated, adult male intruder (either intact or castrated depending on the test) was placed in a female's cage for 5 min. Intruders were different from the stud males. For virgin females, also caged in pairs, the procedure was identical but there were no pups to take care of.

Every test was video-recorded and an observer blind to the experimental conditions scored the attacks and refusal behaviours towards the intruder during the five-minute test. A refusal is a sudden and vigorous response of the female to a male approach, usually consisting of kicks with the forelimbs or hindlimbs. By contrast, an aggression was scored when the female spontaneously bit, kicked, chased and tail-rattled at the intruder. These behaviours were scored using the event recorder of the video-track software SMART 2.5 (Panlab S.L., Barcelona, Spain).

Statistical analysis was performed using IBM SPSS Statistics 19.0, with the latency to attack and the total time attacking as the dependent variables. We first checked whether the data fulfilled the conditions of an ANOVA, e.g. normality (Kolmogorov–Smirnov's test), homoscedasticity (Levene's test) and sphericity (Mauchly's test). These analyses showed that our data were not normally distributed even after log-transformation. Therefore, we used Log-rank tests (latency) and randomization tests (duration of attacks), since these kinds of tests are more robust and adequate for this type of data (Adams and Anthony, 1996; Good, 2005). This analysis was carried out with R free-software (Development Core Team; 2008, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org).

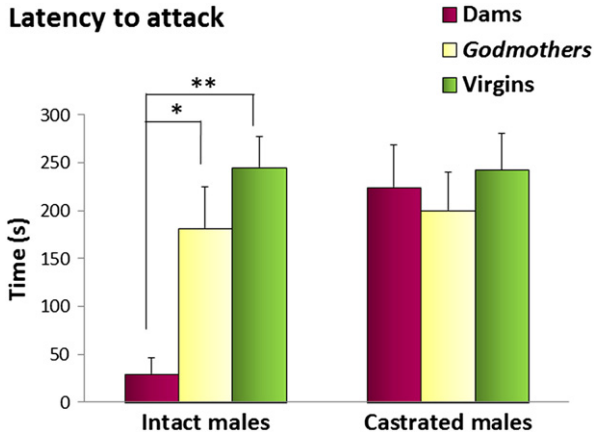

Fig. 1. Latency to attack intact or castrated male intruders by females. Bar histogram illustrating the time (mean ± SEM) that dams (n = 9), *godmothers* (n = 9) and pup-inexperienced virgin females (n = 9) took to initiate an attack on intact (left) and castrated (right) male intruders. Log-rank tests indicated that dams attacked intact male intruders (but not castrated intruders) with a shorter latency than did either virgin females or *godmothers* (*p value < 0.05; **p value < 0.03). A female outlier in the *godmother* group has been excluded from the figure, but not from the statistical analysis.

Results

Latency to attack (Fig. 1). The analysis of the attack latency using the Log-rank test shows differential responses of the females as a function of the gonadal status of the male intruder. Thus, the kind of female has no significant effect on the latency when the intruder is a castrated male ($\chi^2 = 1.7$ on 2 df, $p = 0.432$), but the effect of female is highly significant when the intruder is an intact male ($\chi^2 = 9.8$ on 2 df, $p = 0.00762$). Post-hoc analysis of this effect confirms differences between dams and *godmother* females ($\chi^2 = 4$ on 1 df, $p = 0.0452$), and between dams and virgin females ($\chi^2 = 8.1$ on 1 df, $p = 0.00452$). On the other hand, there are no differences between *godmother* and virgin females ($\chi^2 = 0.5$ on 1 df, $p = 0.47$).

Total duration of attack (Fig. 2). The data are not normally distributed and furthermore the homoscedasticity hypothesis is not tenable even after a logarithmic transformation. Therefore, comparison of the total

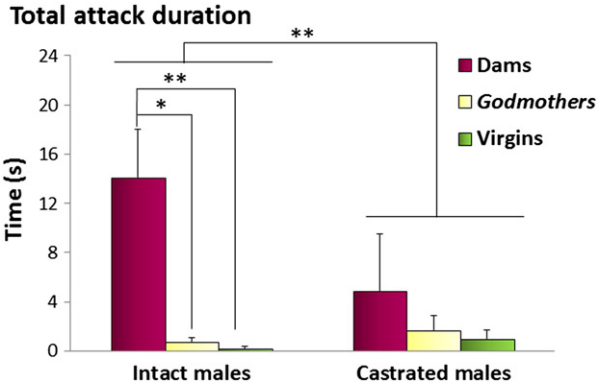

Fig. 2. Total attack duration of females on intact or castrated male intruders. Total time (mean ± SEM) that dams (n = 9), *godmothers* (n = 9) and virgin pup-inexperienced females (n = 9) spent attacking intact (left) and castrated (right) male intruders. Randomisation statistics indicate that dams (but not virgin females, irrespective of their contact with pups) attacked intact male intruders for a longer time than castrated male intruders (randomization tests, *p value < 0.05; **p value < 0.01). A female outlier in the group *godmother* has been excluded from the figure, but not from the statistical analysis.

duration of attack to intact and castrated males by the different types of females was performed by means of a randomization test. Briefly, with the original observations on attack duration we generated 999 additional data sets by permuting the correspondence between each female–male confrontation and the group of females (dams, *godmothers* and virgin females). The F statistic (used in ANOVA) is evaluated for the original data (F_1) and for each randomly generated data set F_2–F_{1000}. Under the null hypothesis of no difference among groups F_1 is distributed as the F_2 to F_{1000} and any order of F_1–F_{1000} values has the same probability, i.e. any rank for F_1 in the series is equiprobable. If r_1 is the rank of F_1 value in the series of F values F_1–F_{1000}, the p-value for a two-tailed test is calculated as the fraction of $2 * (r_1 / 1000)$ (if $r_1 < 500$) or $2 * (1000 - r_1 / 1000)$ (if $r_1 > 500$).

The results of this analysis indicate that females behave similarly when the intruder is a castrated male ($p = 0.256$), but attack duration to intact intruders significantly differs among females ($p = 0.002$). This was further explored by pairwise comparisons: dams attack more than *godmothers* ($p = 0.001$) or virgin females ($p < 0.001$), but no differences were found in the time of attack between virgin and *godmother* females ($p = 0.215$). Thus, lactating females attacked intact males more than virgin females, which showed similar attack duration irrespective of their experience with pups.

These results lead to two main conclusions. First, only dams attack intruders and, more importantly, *godmothers* and pup-inexperienced virgin females show identical behavioural response towards intruders, i.e. virtually no attacks. This clearly indicates that continuous, close contact with the pups (specifically pup-derived odours, see discussion above) and the presence of a nest to defend in the home cage, are not sufficient conditions to induce attacks to intruders (maternal aggression). Therefore our data demonstrate the causal relationship between physiological processes related to pregnancy, parturition and lactation and the induction of maternal aggression. The second conclusion derived from our results is that lactating dams attack intact much more than castrated male intruders, thus replicating previous observations in CD1 mice (Rosenson and Asheroff, 1975). In the context of chemical communication this supports the ideas discussed above that chemosignals reflecting maleness are potent cues that elicit attacks from lactating dams.

Experiment 2: aggression-promoting properties of recombinant darcin in lactating females

To test this, twenty virgin adult females were randomly assigned to two groups: dams ($n = 10$) and *godmother* females ($n = 10$), which were treated as in experiment 1. These females were tested for maternal aggression using castrated males as intruders ($n = 48$), whose testes were surgically removed as in experiment 1. For the aggression tests, castrated male intruders were randomly assigned to one of three groups, which were swabbed in the neck and anogenital region with one of three different stimuli ($n = 12$ per treatment group): animals in the control group were sprayed with 10 μl of phosphate buffer in each zone (PB 0.05 M, pH 7.6); in the r-darcin group, males were rubbed with 10 μl of r-darcin in each zone (1 mg/ml in PB 0.05 M pH 7.6). In the urine group, castrated males were swabbed with 5 μl of intact complete male urine from Swiss albino mice in each zone (Janvier Labs, France). Aggression tests were performed and measured as described for experiment 1. Each female was confronted with a castrated male intruder from the control, darcin and urine groups, in three aggression tests performed over three consecutive days, corresponding to PPD 3–5 for the dams. The order of male presentation was counterbalanced for each group of females. As in experiment 1, each male was used in no more than two tests and never used twice with a lactating dam, to avoid damage and the possible influence of male experience on the outcome of the test. Tests were video-recorded and the attacks were registered from the video by a person blind to the experimental conditions using SMART 2.5 (Panlab S.L.), as in experiment 1.

Male urine was provided by Janvier Labs from healthy, adult stud mice of the strain CD1–ICR. Recombinant darcin was obtained by heterologous expression of the recombinant protein in *E. coli* according to Roberts et al. (2010), where the reader is referred for further details. The recombinant pheromone has been demonstrated to be biologically active: it is attractive to females and is able to endow male-derived odorants with secondary attractive properties (Roberts et al., 2010), and it induces spatial learning in females (and males) (Roberts et al., 2012), thus indicating that it has some sort of rewarding properties. Our experiment is thus designed to test if the same chemosignal is involved in attraction and aggression in females of different reproductive state.

Data were analysed with SPSS. Since the data (latency to attack, total duration of attacks) did not comply with the requirements of an ANOVA, they were log-transformed (Log x + 1). Log-transformed data could be analysed using a 2 × 3 ANOVA, with FEMALE (dams and *godmothers*) as an inter-subject factor and STIMULUS (saline, r-darcin and urine) as an intra-subject factor. The ANOVA was followed by post-hoc pairwise comparisons with Bonferroni p-value corrections.

Results

Latency to attack (Fig. 3). The results of the ANOVA revealed highly significant effects of both FEMALE ($F_{1,18} = 17.303$, $p = 0.001$) and STIMULUS ($F_{2,36} = 3.762$, $p = 0.033$), but no interaction between these factors ($p > 0.4$). A detailed analysis of these results indicates that dams attacked with much shorter latency (mean ± SEM; 23.21 ± 19.64 s) than *godmothers* (140.82 ± 19.35 s). On the other hand, post-hoc analysis of the main effect of STIMULUS indicates that females showed a significantly longer latency to attack saline-sprayed castrated males than those swabbed with urine ($p = 0.018$), whilst r-darcin rendered attack latencies that were more similar to male urine ($p = 1.0$) than to saline ($p = 0.072$, see Fig. 3). These results indicate that females, whether lactating dams or *godmothers*, discriminated between the three kinds of stimuli, with r-darcin eliciting a response similar to urine. Moreover, as in experiment 1, dams readily attacked male intruders, whereas *godmothers* displayed a very long latency to attack.

Total duration of attack (Fig. 4). The repeated measures ANOVA comparing the total duration of attack between FEMALES (*godmothers* and dams) and STIMULI (control, r-darcin and urine) showed a significant main effect of FEMALE ($F_{1,18} = 134.41$, $p > 0.001$) and STIMULUS ($F_{2,36} = 8.59$, $p = 0.001$) but, again, no interaction between these factors ($p > 0.4$). Results indicated that both types of females discriminate

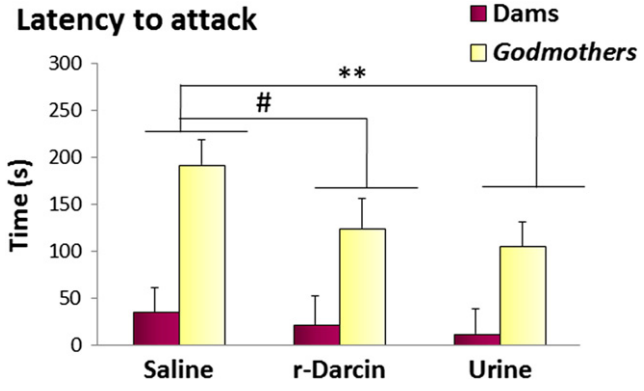

Fig. 3. Male chemosignals reduce latency to attack in dams. Time (mean ± SEM) that dams ($n = 10$) and *godmothers* ($n = 10$) took to initiate an attack on castrated males that were swabbed with phosphate buffered saline, r-darcin and urine of gonadally intact males. There was a highly significant effect of the FEMALE, with dams attacking much earlier than *godmothers* (repeated measures ANOVA of log-transformed data, see text). In addition, male urine and r-darcin strongly reduce latency as compared to phosphate buffered saline (p-values derived from ANOVA and post hoc analysis of log-transformed data; #$p = 0.072$; **$p < 0.01$).

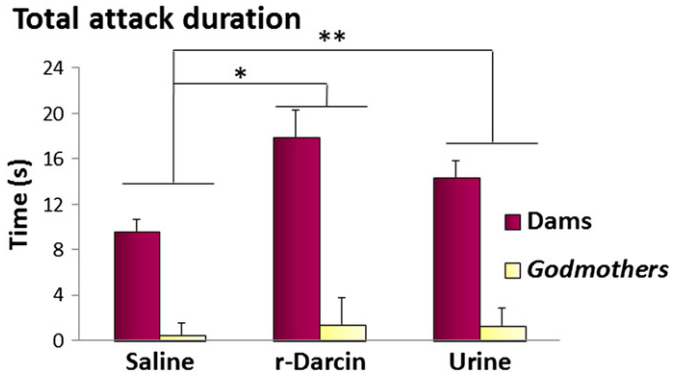

Fig. 4. Maleness chemosignals increase total duration of attack to castrated male intruders by females. Total time (mean ± SEM) that dams (n = 10) and *godmothers* (n = 10) attacked castrated intruders that were swabbed with phosphate buffered saline, r-darcin and urine of gonadally intact males. Dams attacked for much longer (≈×12) than *godmothers*, whilst urine- and darcin-swabbed castrated male intruders were attacked similarly, and much more than saline-treated controls (p-values derived from repeated measures ANOVA and post hoc analysis of log-transformed data:*p < 0.05; **p < 0.01).

chemosignals between different types of castrated male intruders in the same manner. However, our data clearly show that lactating females attacked intruders for much longer than did *godmothers* (>12 times more). Pairwise comparisons of the different STIMULI, using Bonferroni correction, indicated that females spent more time attacking intruders swabbed with urine (p = 0.001) or with r-darcin (p = 0.014) than with phosphate buffer. In addition, the total duration of attacks was very similar towards castrated males swabbed with urine or r-darcin (p = 1).

In conclusion, our data demonstrate that darcin is an aggression-promoting chemosignal for lactating females. In fact, when applied to castrated male intruders, r-darcin induces the same level of maternal aggression that full urine of an intact male does, even if darcin is odourless or, in the conditions of our test, binds odorants from the castrated male.

General discussion and conclusions

The results of the experiments reported above shed some light on the nature of maternal aggression and on its underlying neuroendocrine substrate. Since maternal aggression is severely reduced in mutant mice having olfactory (Cnagα2$^{-/y}$, Mandiyan et al., 2005; AC3$^{-/-}$, Wang and Storm, 2011) or vomeronasal dysfunction (Leypold et al., 2002; Kimchi et al., 2007; Hasen and Gammie, 2009; Chamero et al., 2011; Leinders-Zufall et al., 2014), it seems likely that exposure to pup chemosignals detected by both the olfactory and vomeronasal epithelia is necessary for the induction of maternal aggression. However, our results indicate that virgin females that have been continuously exposed to pups for 3–4 days show negligible aggressive behaviour towards intruders approaching the nest (from which pups had been previously removed). Therefore, in our experimental conditions, chemosignals only induce full nest defence in females that have undergone pregnancy, parturition and lactation.

In this respect, early work by Svare and Gandelman (1976) indicated that suckling stimulation was required for the development of maternal aggression. In fact, these authors were able to develop aggression in virgin females that were in contact with pups provided that pups attached themselves to the nipples. Development and growth of the nipples in virgin females were achieved by means of daily injections of oestradiol and progesterone for 19 days. Although this treatment might, itself, contribute to maternal aggression induction, thelectomy (surgical removal of the nipples) strongly reduced aggression in both parturient females and hormone-treated virgin females that had close contact with pups.

Taken together, these results suggest that suckling and chemosensory stimulation both contribute to the induction of maternal aggression in mice. However, female aggression starts a couple of days before parturition in both mice and rats (see Mann et al., 1984; Bosch, 2013), suggesting an additional role of endocrine factors acting during late pregnancy. Studies in rats indicate that pup-directed maternal behaviours are promoted by the action of prolactin onto a substrate of high oestradiol circulating levels after progesterone withdrawal (Bridges and Ronsheim, 1990). Nevertheless, female laboratory mice show spontaneous maternal behaviour without apparent need for endocrine induction (Stolzenberg and Rissman, 2011), and the roles played by steroids (e.g. Mann et al., 1984) and prolactin in murine maternal aggression are far from clear (Mann et al., 1980; Svare et al., 1982). Brain oxytocin is well known to play a pivotal role in the control of maternal aggression (see Bosch, 2013). Nonetheless, the limited permeability of the blood brain barrier for oxytocin (Ermisch et al., 1985) makes it unlikely that the raising of circulating levels of this hormone during parturition and suckling might have a direct effect on behaviour. Future studies will help to understand how these three factors – chemosignals, nipple stimulation and hormones – interact to induce nest defence in lactating dams.

A second question tackled in this study is the existence and identity of maternal aggression-promoting chemosignals in mice. In our experiments, dams are extremely aggressive against intact male intruders, but less so when intruders were castrated. This indicates that some maleness signal(s) promote or enhance aggression to male intruders in dams, which is nevertheless expressed to every kind of conspecific intruder including castrated males and intact or ovariectomised females (Rosenson and Asheroff, 1975). Although the results of our two experiments are consistent in that point, dams' aggression towards castrated males apparently differs between experiments 1 and 2. Thus attacks occur earlier (latency 224.73 ± 42.30 s in experiment 1; 35.60 ± 26.12 s in experiment 2) and for a longer time (4.89 ± 4.56 s in experiment 1; 9.66 ± 1.00 s in experiment 2) in the second experiment than in the first one. We have not analysed statistically this difference, because experiments were not run in parallel and, even if the conditions were similar, many variables may affect aggressiveness. For instance, running a pup-retrieval test just before the maternal aggression test results in a high rate of attacks to both intact and castrated male intruders (unpublished results). This is an interesting issue that requires further investigation.

Concerning the nature of the maleness chemosignals enhancing maternal aggression, our findings demonstrate that darcin is able to induce aggression at a level similar to full urine. This has two important implications. First, darcin is a urinary protein involved in both attraction and aggression. Although thiazoline is a urine-borne ligand for darcin, the protein alone (recombinant, devoid of testosterone-dependent urinary volatiles) is able to induce both behaviours in female mice. Being a large protein, of nearly 19 kDa molecular weight, darcin is probably detected by V2R in the VNO.

Our results also indicate that the change in behavioural response towards males that takes place around parturition does not consist of a modification of chemosensory organ response to chemosignals. Thus, as discussed in the Introduction, a possible explanation for a change of response to conspecific chemosignals could be a specific change in the pattern of receptor expression in the vomeronasal and/or olfactory epithelia (e.g. Alekseyenko et al., 2006) or inhibition of the responsiveness of olfactory and/or vomeronasal sensory neurons to their ligands (Stowers and Dey, 2014). However, as the same ligand, darcin, induces two different responses in females of different reproductive status, the most parsimonious explanation for this change is that the same receptor detects darcin throughout female's life, but it is the behavioural response to this that changes. This probably occurs through modifications of the socio-sexual brain, which could be induced by a mixture of endocrine agents and sensory inputs (chemosignals and, possibly, nipple stimulation) acting around the time of parturition.

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