Behavioural Brain Research 200 (2009) 277–286

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Refining the dual olfactory hypothesis: Pheromone reward and odour experience

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ARTICLE INFO

Article history:
Received 30 May 2008
Received in revised form
23 September 2008
Accepted 1 October 2008
Available online 11 October 2008

Keywords:
Vomeronasal
Intersexual attraction
Learning
Reinforcement
Mice

ABSTRACT

In rodents, sexual advertisement and gender recognition are mostly (if not exclusively) mediated by chemosignals. Specifically, there is ample evidence indicating that female mice are 'innately' attracted by male sexual pheromones that have critical non-volatile components and are detected by the vomeronasal organ. These pheromones can only get access to the vomeronasal organ by active pumping mechanisms that require close contact with the source of the stimulus (e.g. urine marks) during chemoinvestigation.

We have hypothesised that male sexual pheromones are rewarding to female mice. Indeed, male-soiled bedding can be used as a reinforcer to induce conditioned place preference, provided contact with the bedding is allowed. The neural mechanisms of pheromone reward seem, however, different from those employed by other natural reinforcers, such as the sweetness or postingestive effects of sucrose.

In contrast to vomeronasal-detected male sexual pheromones, male-derived olfactory stimuli (volatiles) are not intrinsically attractive to female mice. However, after repeated exposure to male-soiled bedding, intact female mice develop an acquired preference for male odours. On the contrary, in females whose accessory olfactory bulbs have been lesioned, exposure to male-soiled bedding induces aversion to male odorants. These considerations, together with data on the different properties of olfactory and vomeronasal receptors, lead us to make a proposal for the complementary roles that the olfactory and vomeronasal systems play in intersexual attraction and in other forms of intra- or inter-species communication.

1. Introduction: the role of sexual pheromones in sexual reproduction

Sexual reproduction has important long-term evolutionary advantages related to the generation of genetic diversity [71]. However, sex requires a strong investment of energy and resources mainly in the production of a relatively reduced number of yolk-laden eggs by the females (that therefore constitute the limiting sex) and in the search of good-quality mates [85]. Strategies for maximizing the probability of mating with reasonable energy costs include sexual advertisement, namely behaviours aimed at informing possible mates of one's availability to mate (as well as of one's quality as a potential mate). To do so, some species (most birds and primates) use visual or auditory stimuli. Although this can be quite effective, it is not riskless. Visual displays and courtship songs are not only energetically expensive but can attract predators. In contrast, other species rely on chemicals released to the environment to attract possible mates, which are usually called sexual pheromones. In some cases, tiny amounts of pheromones can be effective, making sexual advertisement cheap enough to be affordable for the limiting sex, like in some moths (see [85]). In contrast, some male rodents excrete a huge amount of a kind of proteins usually considered pheromone reservoirs [77], the so-called Major Urinary Proteins (MUPs), which are present in male urine at concentrations as high as 30 mg/ml (see [11]). In addition, male mice spend a considerable amount of time and energy in maintaining fresh urine marks in critical points of their territory [30]. These immense costs of chemical communication are probably worth because they efficiently provide a clear message to conspecifics (possible mates and competitors), while being not conspicuous to most predators, which might detect urine marks but have serious difficulties to locate their owners [2].

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doi:10.1016/j.bbr.2008.10.002

The effectiveness of pheromones as sexual advertisers relies, besides other general properties of chemosignals (e.g. stability in time, detectability at a distance and through some physical barriers or in the darkness), on their attractiveness for potential receivers (possible mates). Indeed, this feature caused the identification of the firsts sexual pheromones in insects and promoted the concept of pheromones [34]. Some male moths respond with sudden changes in flight direction (upwind flight) to the detection of few molecules of female pheromone [70]. This powerful response to pheromones immediately excited the imagination of both the lay public and scientists, about the idea that mammals, and even human beings, might have similar sexually arousing chemicals. However, the evidence supporting the existence of attractive sexual pheromones in mammals, not to say in humans, is not abundant.

2. Do mammals have sexual pheromones? The importance of considering experience

As we have already discussed, rodents are among the most studied macrosmatic mammals and their urine (e.g. male mice) is extraordinarily rich in molecules excreted for no other apparent purpose than communication. Therefore, one would expect that most rodents display conspicuous pheromone-elicited responses. Indeed, female of both rats and mice show several neuroendocrine responses to urine of conspecifics, strongly suggesting that rodents excrete or secrete priming pheromones [26]. In mice, these responses include failure in embryo implantation induced by urine of strange males (the so-called Bruce effect), oestrus arrest induced by overcrowding in females, estrous induction by male urine or accelerated sexual maturation induced in prepubertal females by male urine (for a review, see [25]).

In the same line, there is evidence that many rodents have releaser pheromones, excreted or secreted molecules causing immediate changes in behaviour, such as sexual pheromones. Thus, it has been repeatedly reported in different rodent species that adult specimens preferentially explore urine or bedding soiled by adults of the other gender (see studies in mice [20]; rats [66]; guinea pigs [7] and hamsters [65]). In addition, it is well known that males' behavioural responses towards conspecific intruders are gender specific: they readily attack male intruders (inter-male aggression) but display 70-kHz courtship songs [28,59] and try to mount females. Attack responses from a resident male can be induced easily towards 'neutral' intruders (e.g. castrated males) by simply spraying them with urine of intact males (see [80]). In addition, male mice display typical ultrasonic vocalizations towards female, but not male urinary cues [61], in their very first experience with these stimuli [24], even if this behaviour is modulated by experience [24,39].

Although these data might seem a solid evidence of the presence of sexual pheromones in rodents, this kind of responses may be the result of previous experience. According to the classical definition [34], for a secreted substance to be a pheromone, it must elicit stereotyped responses in conspecifics, that is, responses that are not learned but innate or unconditioned (see discussion in [50]). In other words, the presence of a detectable behavioural response to chemicals derived from conspecifics of the other gender is not a demonstration that these chemosignals constitute a pheromone. Indeed, as male and female animals gain sexual experience, a cohort of stimuli related with their sexual partners may become associated with sex. By means of associative learning these mate-derived stimuli, including odorants, may become secondarily attractive. For instance, pairing a synthetic odorant with paced mating in females makes them to prefer males scented with this odorant [17]. Mice may develop other kinds of conditioned responses towards 'mate-derived' odorants. Thus, it has been shown that male mice can be induced to emit ultrasonic vocalisations to female rat's urine by exposing them to female mice sprayed with urine of female rats [39]. Furthermore, if males are allowed to interact with females that have ingested important amounts of the spice fenugreek, they start vocalising when fenugreek is presented alone [39]. The same kind of conditioned responses might occur toward odorants actually secreted by the animals, which would then become attractive odorants but, even so, could not be considered pheromones.

3. Sexual pheromones in mice: innately attractive chemical signals

On this framework we decided to check whether the attraction that adult mice show for conspecifics of the other gender was innate or learned, by testing if young adult mice without any experience with adults of the other gender were attracted by chemical stimuli derived from them. Thus, we reared females in a room with no adult males. Pregnant females were placed in a clean room without males and, 19 days after delivery, male youngsters were removed [57]. Their female siblings, which we call '*chemically naïve*' females, were used in a series of tests to analyse their behavioural response to chemosignals from different kinds of conspecifics, as well as to synthetic odorants.

Two kinds of tests were performed (Fig. 1A and B). In the volatile plus non-volatile two-choice preference tests (V + NV test), the females were introduced in a rectangular test cage with two dishes located on opposite sides of the cage, containing the stimuli (Fig. 1A). The exploratory behaviour of the females was video-recorded for 5 min and the time that the animals spent sniffing the dishes (at less than 1 cm from them) was measured by a person who was unaware of the stimuli present in the dishes and the condition of the animals. In some of our experiments, the position of the animal was determined by means of video-tracking software (SMART, Panlab, Cornellà del Vallés, Spain) and the time the animals spent next to each dish automatically measured (Fig. 1C and D). In the second kind of tests, the two-choice preference test for volatiles (V test), an evenly perforated methacrylate platform (holes of 3 mm in diameter every 2 cm) was located on top of the dishes so that contact with the bedding was effectively prevented, although animals could sniff through the holes thus detecting the volatiles emanating from the bedding (Fig. 1B). We video-recorded the behaviour of the females during 5 min and measured the time that animals explored each dish using the same procedures as described for the V + NV tests.

In our experiments we first perform a control test in which typically female- (F/F) or castrated male-soiled bedding (CM/CM) was present in both dishes. This control test allows us to exclude animals showing a clearly unbalanced exploratory behaviour, thus reducing variability and the number of animals employed. To do so, we calculate a preference ratio for the right-side dish ($Rr = [right/(right + left)]$) and only those females for which $0.33 < Rr < 0.66$ are used in subsequent tests. These females are then run in a male vs. female (M/F) or male vs. castrated male (M/CM) test. The preference for male-derived stimuli is investigated by analysing the results of both the control (e.g. F/F) and the male preference test (e.g. M/F) using a two-way ANOVA for repeated measures, with test and side as within subject variables. The presence of a significant interaction between both variables is the result that indicates a substantial preference for male-derived chemosignals. This analysis is more demanding than a simple test comparing left vs. right sides.

These experiments have revealed that CD-1 chemically naïve females show no preference for volatiles derived from male-soiled bedding in V tests in which this stimulus is confronted to female-soiled bedding [57] or to castrated male-soiled bedding [52,53].