Animal Behaviour 97 (2014) 265–272

Contents lists available at ScienceDirect

Animal Behaviour

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

Special Issue: Biochemistry & Animal Communication

Sex pheromones are not always attractive: changes induced by learning and illness in mice

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ARTICLE INFO

Article history:
Received 12 April 2014
Initial acceptance 16 May 2014
Final acceptance 22 July 2014
Available online 9 September 2014
MS. number: 14-00300R

Keywords:
illness cues
learning
maternal aggression
olfactory
puberty
sexual attraction
vomeronasal

A male-specific major urinary protein named darcin is attractive to female mice, *Mus musculus*, stimulates a learned attraction to volatile components of a male's urinary odour and induces spatial learning. In this article we show that darcin also induces learned attraction for a previously neutral olfactory stimulus (the odorant isoamyl acetate), acquired by repeated presentation of both stimuli together. We hypothesize that this is a case of olfactory–vomeronasal associative learning, in which darcin acts as the unconditioned reinforcer. However, the presence of darcin is not always attractive to adult female mice. Urine from males parasitized by the nematode *Aspiculuris tetraptera* has no attractive value for females, despite apparently normal presence of darcin. The loss of attractive value may be due to unknown infection-derived chemicals whose detection overrides the attraction induced by darcin, or prevents detection of darcin by other (unknown) mechanisms. Other cases in which male urine (and thus the presence of darcin) does not induce attraction are discussed, namely in lactating females, which respond with aggression towards intruder males, and in prepubertal females, which show aversive responses towards unfamiliar male urine. Thus, although the darcin sex pheromone induces attraction in adult female mice that does not need to be learned, such innate pheromonal responses can be modulated by the physiological or health status of the sender and receiver. This provides a degree of flexibility in response to pheromonal signals, but such that individuals of the same class or status still share consistent predictable responses to improve their reproductive fitness. Further, by readily inducing the same response towards other odorants through associative learning, pheromones can also target responses flexibly towards odour signatures at an individual-specific level.

Pheromones were originally defined as 'substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process' (Karlson & Luscher, 1959, p. 55). Although this definition has been very useful for more than 50 years (Wyatt, 2009), the response to pheromones (at least in mammals) may vary depending on a number of factors, including the previous experience or the hormonal status of the receiver (Wyatt, 2010). In rodents, in which olfactory stimuli play a key role in many aspects of sociosexual behaviours (Brennan & Kendrick, 2006), experience with social chemical signals can influence later responses. For example, chemical signals present in urine of male mice, *Mus musculus*, detected through the vomeronasal system, have reinforcing properties able to induce appetitive associative learning, in such a way that other volatiles present in urine may become secondary attractive odorants (Martinez-Ricos, Agustin-Pavon, Lanuza, & Martinez-Garcia, 2007, 2008; Moncho-Bogani, Lanuza, Hernandez, Novejarque, & Martinez-Garcia, 2002; Ramm, Cheetham, & Hurst, 2008). Therefore, the behavioural response elicited by pheromones may also be seen as a learned response to stimuli previously associated with the pheromones, so that these stimuli acquire pheromone-like properties (Martinez-Garcia et al., 2009). Recently, a male-specific urinary protein named darcin has been shown to be able to induce this kind of olfactory learning (Roberts et al., 2010) and also spatial learning (Roberts, Davidson, McLean, Beynon, &

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http://dx.doi.org/10.1016/j.anbehav.2014.08.011
0003-3472/

Hurst, 2012). Since odours are easily associated with either positive or negative experiences (Herz & Cupchik, 1995), we hypothesized that darcin would be able to induce a secondary attraction by association with a neutral odorant (not present in urine), and to test this hypothesis we performed experiment 1, described below.

Notably, though, the behavioural response of females to the reinforcing properties of darcin (and maybe other male sexual pheromones) is affected by their endocrinological status. On the one hand, the preference of female mice for male-derived chemicals has been shown to appear with puberty, whereas prepubertal females display an aversive response to chemical signals from unfamiliar adult males (Drickamer, 1989; Mucignat-Caretta, Caretta, & Baldini, 1998). We do not yet know whether hormonal changes at puberty modify the behavioural response to darcin, changing it from aversion (shown by prepubertal females) to attraction (shown by postpubertal females), or whether other airborne components induce aversion to urine from unfamiliar adult males that prevents contact and delivery of this involatile protein pheromone to receptors. On the other hand, during lactation females show aggressive responses towards unfamiliar male intruders (and to a lesser extent also towards female intruders), to protect their pups (maternal aggression, Rosenson & Asheroff, 1975). Maternal aggression is low towards castrated males, and depends on the vomeronasal organ, as revealed by lesion (Bean & Wysocki, 1989) and gene knockout studies (Chamero et al., 2011). Hence, maternal aggression is elicited by the vomeronasal detection of testosterone-dependent chemical stimuli that may include darcin. Indeed, recent results from our laboratory indicate that the attractive pheromone darcin is also able to induce maternal aggression (Martin-Sanchez et al., 2014). Therefore, the female's hormonal status during lactation induces unknown changes in the neural circuitry processing darcin that alter the behavioural response to this pheromone from attraction to aggression.

In addition to the endocrinological changes at puberty and lactation that alter the behavioural response of females, previous studies have shown that viral infections or parasitosis in male mice used as donors of urine or bedding result in a lack of attractiveness of the urine (or soiled bedding) for females (e.g. Kavaliers, Choleris, & Pfaff, 2005; Penn et al., 1998). This lack of attractiveness may result from a loss of darcin expression as a result of the infection, or to the presence of unknown infection cues in the urine of infected males. To test these possibilities, we performed preference tests (experiment 2) using urine from males parasitized by the nematode *Aspiculuris tetraptera* versus (healthy) female urine, and tested whether the urine of parasitized males contains darcin.

EXPERIMENT 1: INDUCING ATTRACTION FOR A NEUTRAL ODORANT

Methods

Subjects

For the present study, 15 adult female mice (12–16 weeks) of the CD1 outbred strain were used (Janvier Labs, Le Genest-Saint-Isle, Saint-Berthevin Cedex, France). Treatment of these and the other animals used in experiments 1 and 2 complied with the European Union Council Directive of June 3 2010 (6106/1/10 REV1), according to which procedures were approved by the Committee of Ethics on Animal Experimentation of the University of Valencia (protocol number A1283764105250). Procedures also adhered to the ASAB/ABS Guidelines for the Use of Animals in Research.

The females were sexually naïve and had never been exposed to chemical signals from sexually mature males. To achieve this, pregnant females were housed in a clean room without males, in standard macrolon transparent cages with a wire lid (21.5 × 46.5 cm and 14.5 cm high, ref. 1000, Panlab, Barcelona, Spain) filled with soft wood bedding (Souralit S.L., ref. 3000, Barcelona, Spain), provided with nesting material (shredded paper) and enriched with cardboard tubes. The room was maintained at 22–24 °C, 60–80% relative humidity and a 12:12 h light:dark cycle, with lights on at 0800 hours. Food (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan, ref: 2014) and water were available ad libitum. Nineteen days after delivery (well before puberty, which usually takes place about 6 weeks of age, see Silver, 1995), pups were sexed and males were removed. Female siblings were kept in a clean room in complete absence of adult male chemical signals, in groups of five or six per cage (the stock housing conditions in the experimental room were the same as described above for the pregnant females). Food and water were available ad libitum except during the 5 min preference tests and the 15 min training sessions. Welfare assessment took place during cage cleaning, and included noninvasive indicators. In the neonates, skin colour, activity and presence of the milk spot were observed; at weaning and in the adult, general appearance, size, coat condition, posture, gait, activity levels, interaction with the environment and clinical signs were observed (Wells et al., 2006). After weaning, animals were only manipulated for cage cleaning once a week. Since general appearance and size were evaluated as normal, no further care was necessary. Mice were handled following the standard practice of picking them up by gently holding the base of the tail and helping them onto the handler's arm, avoiding holding them in the air. All procedures involved in this study were noninvasive behavioural tests. At the end of the experiments, animals were euthanized with an intraperitoneal overdose of sodium pentobarbital (92 mg/kg), as indicated in the approved protocol (cited above). The male siblings were either used for anatomical studies (protocols approved by the Committee of Ethics on Animal Experimentation of the University of Valencia, under the same reference number A1283764105250; published elsewhere, Otero-Garcia et al., 2014) or euthanized as described above.

Stimuli

We chose two odorants that have frequently been used as neutral olfactory stimuli in the literature: isoamyl acetate (e.g. Angely & Coppola, 2010; Panreac, Barcelona, Spain) and citralva (e.g. Martinez-Ricos et al., 2007; geranonitrile, 3,7-dimethyl-2,6-octadiene-1-nitrile, International Flavours and Fragrances, Ventos, Barcelona, Spain). Both odorants were diluted 1:1000 in phosphate buffer (0.01 M) with 0.01% Triton X-100. In a pilot test of olfactory preference isoamyl acetate and citralva were investigated equally.

Preference tests

Animals were habituated to handling and to the test cage over 3 days, 10 min per day, between 1500 and 2000 hours. Preference tests were performed in cages measuring 25 × 50 cm and 30 cm high. Two 4 × 4 cm pieces of filter paper each impregnated with 5 μl of one of the stimulus odorants were presented on opposite sides of the cage. These impregnated papers were fixed to the bottom of the cage with a metallic cover, leaving exposed a circular area (diameter = 3.5 cm) that allowed direct nasal contact with the paper but prevented the animals gnawing or removing it.

For this olfactory preference test (citralva versus isoamyl acetate) females were released in the centre of the cage, the experimenter left the room, and the behaviour was video recorded for 5 min. The time that the animal spent in the circular area covered by the paper was measured by tracking animals automatically using the video analyser software Smart 2.5 (Panlab, Barcelona, Spain; see Fig. 1a). Since we observed that the animals lost interest in the olfactory stimuli at the end of the test, we restricted the analysis of the data to the first 4 min.