Intraspecific Communication Through Chemical Signals in Female Mice: Reinforcing Properties of Involatile Male Sexual Pheromones

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Abstract

In rodents, social and reproductive behaviors critically depend on chemical signals, including sexual pheromones that have been suggested (but not demonstrated) to be rewarding. In this work, we analyze this issue by studying the chemoinvestigatory behavior of adult female mice (without experience with male-derived chemicals) toward 1) the synthetic odorant citralva, 2) bedding soiled by different conspecifics (females, males, and castrated males), and 3) volatiles derived from bedding soiled by males and castrated males (confronted in 2-choice tests). We also study whether these chemical signals are able to induce conditioned place preference, a reliable test for rewarding properties of stimuli. The results show that involatile, male-derived chemicals elicit an intense and sustained chemoinvestigation and, more importantly, are the only tested chemical signals that induce conditioned place preference. In contrast, volatile, male-derived chemicals are not significantly chemoinvestigated. Bedding soiled by castrated males induces a transient chemoinvestigation, likely directed to steroid-independent, biologically relevant chemical signals, whereas the intense chemoinvestigation of female-soiled bedding shows a slow habituation. Finally, females did not explore significantly citralva-odorized bedding. The present work constitutes the first demonstration of the unconditioned reinforcing properties of involatile (likely detected by the vomeronasal organ) steroid-dependent chemical signals in mammals.

Key words: olfactory system, place preference, reward, sexual behavior, vomeronasal system

Introduction

In rodents, social and reproductive behaviors are critically dependent on communication through chemical signals. These include secreted or excreted chemicals that induce reliably endocrine and/or behavioral responses in conspecifics, thus fitting the classical definition of pheromone (Karlson and Luscher 1959). In different rodent species, some of the sexual pheromones (chemical signals mediating intersexual communication) are detected by the vomeronasal organ (VNO; see Halpern and Martínez-Marcos 2003). Thus, lesions of the VNO of male mice significantly reduce the ultrasonic vocalizations they direct to females and female urine, the effects of the lesions being especially dramatic in sexually inexperienced males (Wysocki et al. 1982). In male guinea pigs, lesions of the VNO result in an extinction-like decline of the chemoinvestigatory behavior of female urine (Beauchamp et al. 1985). These authors suggest that VNO-lesioned animals show an olfactory response

that undergoes extinction due to the absence of VNO-detected pheromones that may act as primary reinforcing stimuli (Beauchamp et al. 1983, 1985).

Although some pheromones are detected by the olfactory epithelium (Distel and Hudson 1985; Hudson and Distel 1986) and the VNO seems to be involved in responding to some odor quality signals (see Johnston 1998), the data reviewed above suggest that, in rodents, some unidentified VNO-detected sexual pheromones may constitute a primary rewarding stimulus that is needed to sustain normal sexual behavior. Although the only attempt to demonstrate such putative reinforcing properties of pheromones in rodents rendered inconclusive results (Coppola and O'Connell 1988), recent studies have reopened this issue. Thus, Moncho-Bogani et al. (2002) demonstrated that female mice show an unconditioned preference for involatile compounds contained in male-soiled bedding (when confronted to female-soiled

bedding in 2-choice tests), but not for volatiles emanating from it. Noteworthy, male-derived volatiles could become attractive to females because of repeated exposure to male-soiled bedding, probably due to a process of associative learning in which the volatiles acquire attractive properties by association with the involatile, innately preferred chemical signals. This is consistent with previous findings by Beauchamp et al. (1982, 1983, 1985) suggesting that pheromones act as unconditioned reinforcers that allow odorants to become conditioned reinforcers. In addition, Fos activity indicates that male-derived nonvolatile chemicals strongly activate the vomeronasal and the reward systems of the brain of females (namely the basolateral amygdala and the shell of the nucleus accumbens; see Baxter and Murray 2002), thus strongly supporting the view that VNO-detected, male sexual pheromones are reinforcing to female mice (Moncho-Bogani et al. 2005). Finally, male mice lacking a cluster of vomeronasal receptor genes lack the experience-dependent gain in sexual activity observed in the wild-type mice (Del Punta et al. 2002), a result that the authors interpret as "consistent with the proposed view of the VNO as intrinsically rewarding."

The aim of this work is to study whether involatile male-derived chemicals are rewarding for female mice. To achieve this goal, in Experiment 1, we analyze the chemoinvestigatory behavior of adult female mice (with no previous experience with chemicals derived from adult males) in preference tests in which the females choose between clean bedding and 1) a synthetic odorant, 2) bedding soiled by other female mice, 3) bedding soiled by castrated males, and 4) bedding soiled by intact adult males. These tests are repeated for several days to study the behavioral habituation of the chemoinvestigation elicited by all 4 stimuli. In Experiment 2, we test whether chemical signals derived from different conspecifics are able to induce a conditioned place preference, a well-known behavioral paradigm to check the reinforcing properties of stimuli (Tzschentke 1998). Experiment 3 analyzes whether the learned place preference induced by male-soiled bedding decreases in the absence of the stimulus. Finally, in Experiment 4, we assess whether the male-derived chemical signals that are able to induce place preference are nonvolatile, and thus probably VNO dependent.

Materials and methods

Animals

Experimental animals ($n = 111$) were adult (more than 9 weeks of age), gonadally intact CD-1 female mice reared in the absence of adult males or their derived chemicals. Briefly, pregnant females were housed in a clean room without males. Nineteen days after delivery (early before puberty) pups were sexed, males removed, and the females kept in the same room until the age of 9 weeks (Moncho-Bogani et al. 2002). Animals were treated according to the EEC guidelines for European Communities Council Directives of 24th November 1986 86/609/EEC. Procedures were approved by the Committee of Ethics on Animal Experimentation of the University of València.

Experimental design

All tests were performed in rectangular clear methacrylate cages (25 cm wide, 50 cm long, and 30 cm high) with 2 glass dishes (6 cm diameter and 5.5 cm high), containing about 13 g of clean, soiled, or odorized bedding, which were located in opposite sides of the cage. For Experiment 4, a perforated cover prevented the contact with the bedding but allowed the detection of volatile molecules emanating from it.

Three to four days before each experiment, animals were habituated by placing them in the test cage for 10–15 min daily. Every test was video recorded and an observer blind to the experimental condition measured the time that animals spent exploring each dish (including an area of 1 cm around them) during the first five min of the test. In Experiment 1 (chemoinvestigatory behavior), only the time that the females spent with the nose directed to the bedding was measured, whereas in Experiments 2, 3, and 4 (place preference tests), we measured the time females spent in the defined area around the dish irrespective of the behavior they were displaying.

All the experiments started with a control test to check whether the animals displayed a balanced exploration of the test cage. Those animals that spent twice as much time investigating one of the dishes than the other in this control test were discarded ($n = 10$).

Bedding soiled by conspecifics was obtained from home cages containing 3–6 animals for 4 days, except for the bedding soiled by intact males. In this case, the dominant males of several cages were housed individually to avoid intermale aggressions during the time of bedding collection. To ensure that bedding soiled by a given kind of conspecifics was chemically homogeneous throughout the experiment, bedding from several cages containing the same kind of animals was mixed and stored at $-20\ °C$ until the day of the test. To obtain castrated male–soiled bedding, 3 adult males were orchydectomized under deep pentobarbital anesthesia (55 mg/kg weight, intraperitoneal injection). When fully recovered (1 month after surgery), bedding soiled by them was collected as explained above. Because the experiments lasted several days, stimulus bedding was replaced daily before the start of each session.

Experiment 1

Animals were randomly assigned to 4 groups that were exposed to bedding soiled by females from other cages ($n = 9$), males ($n = 10$), castrated males ($n = 10$), and bedding scented with citralva (geranonitrile, 3,7-dimethyl-2,6-octadiene-1-nitrile, kindly provided by International Flavours and Fragrances, Barcelona, Spain; 2 µl in 20 g of clean bedding;

$n = 10$), respectively. After habituation (with clean bedding in both dishes), females were tested in the control condition (clean vs. clean bedding; Day 0). Then, females were exposed to four 2-choice tests (one trial per day; Days 1–4) of chemo-investigatory behavior, with clean bedding in one dish and soiled or scented bedding in the other one. The location (left or right) of the stimulus on the test cage was changed every test day to avoid association of the stimulus with the location where it was presented.

Experiment 2

In order to test the reinforcing properties of chemical cues from different kinds of conspecifics, we tested whether these chemical cues induced a conditioned preference for the place where they were systematically presented, using an operant place-conditioning paradigm (adapted from Crowder and Hutto 1992). In this paradigm, during the training sessions the animals are free to visit both compartments of the test cage. To obtain the reinforcer, animals should actively explore the dish containing soiled bedding. This differs from the situation in the classic conditioned place preference paradigm, in which the subject is placed in either the reinforced or nonreinforced compartment by the experimenter and, therefore, there is no instrumental behavior being reinforced. Several landmarks within the test room allowed the animals to differentiate between both sides of the cage.

In this experiment, 3 groups of animals ($n = 10$ per group) were exposed to female-soiled bedding (group ''Female''), bedding soiled by castrated males (group ''Castrated Male'') and male-soiled bedding (group ''Male''), respectively. Following the habituation and the control test (clean bedding in both plates, Day 0), animals were run in four 2-choice training sessions (Days 1–4, one session per day) in which bedding soiled by conspecifics was systematically placed in the right ($n = 5$ per group) or the left ($n = 5$ per group) dish, the other dish containing clean bedding. The females were left to explore the cage for 10 min, and their behavior during the first 5 min was recorded for subsequent analysis. On Day 5, we checked whether females had developed a conditioned preference for the place where the stimulus was presented using clean bedding in both dishes (place preference test). Data from the control, 3 training days, and from the place preference test were analyzed.

Experiment 3

This was similar to Experiment 2, but after the place preference test, animals ($n = 16$) underwent 3 additional tests of the exploratory behavior with clean bedding in both dishes (C/C_1, C/C_2, and C/C_3; days 8, 11, and 14). As in Experiment 2, in half of the animals, the male-soiled bedding was presented on the right side of the cage, whereas for the other half, it was located in the left side. Data from the control, place preference test, and the 3 subsequent C/C tests were analyzed.

Experiment 4

In this experiment, we assessed whether the reinforcing chemical signals contained in male-soiled bedding are volatile or involatile. To do so, animals were randomly assigned to 2 groups. In the first group (''Volatiles + non-volatiles,'' V + NV; $n = 7$), direct contact with the soiled bedding allowed access to volatile and nonvolatile substances it contained. In the other group (''Only Volatiles,'' V; $n = 9$), a perforated cover prevented contact with the bedding, so that females could only detect the volatiles emanating from it.

After habituation (castrated male–soiled bedding in both plates), females were run in a control test (castrated male–soiled bedding in both dishes, Day 0) and then in four 2-choice training sessions (Days 1–4, one session per day), in which bedding soiled by intact males was systematically placed in one dish and bedding soiled by castrated males in the other. In each group, the right or left location of male-soiled bedding was counterbalanced. On day 5, we checked whether females had developed a conditioned preference for the place where castrated male– or intact male–soiled bedding were presented using castrated male–soiled bedding in both dishes (place preference test). Data from the control, 3 training days, and from the place preference test were analyzed.

Analysis of the data

A Kolmogorov–Smirnov test (with Lillieford's correction) was used to check whether the data followed a normal distribution. A Student's t-test for paired samples was used to compare the time spent exploring both dishes in the control tests. Data from the preference tests were analyzed using repeated measures analysis of variance (ANOVA). When sphericity could not be assumed (Mauchly's test), the Greenhouse–Geisser correction was applied. When appropriate, the ANOVA was followed by the analysis of the simple effects of the factors and multiple pairwise comparisons adjusted with Bonferroni's correction (see details for the analysis of each experiment in Results). The effect of the location of the stimulus (right or left dish) on the exploratory behavior was assessed by means of a Student's t-test for independent samples (Experiments 2 and 3) or a univariate ANOVA (Experiment 4, in which each subgroup had a different number of animals). All analyses were done using the SPSS 12.0.1 software package.

Results

Experiment 1: dynamics of the chemoinvestigation of conspecific-derived and neutral odorants

In all groups, a Student's t-test showed no difference on the time spent investigating the left and right dishes in the control test ($P > 0.2$ in all 4 groups). A 2-way ANOVA for repeated measures comparing the exploration of the soiled (or

odorized) bedding with GROUP (Female, Castrated Male, Male, and Citralva) as between-subject factor and TEST as intrasubject factor (Days 1–4) revealed a significant GROUP × TEST interaction ($F_{12,140} = 3.393$, $P < 0.001$), as well as significant main effects of the GROUP ($F_{3,35} = 11.445$, $P < 0.001$) and the TEST ($F_{4,140} = 32.249$, $P < 0.001$). Thus, the groups show different patterns of chemoinvestigatory behavior along the 4 days of exposure to odorants.

Multiple pairwise comparisons revealed that the time spent investigating the citralva-scented bedding in each one of the test days was not significantly different from the time spent investigating the clean bedding in the control situation ($P > 0.05$ in all cases, Figure 1A), whereas investigation of castrated male–soiled bedding differed from the control only in the first test day ($P < 0.001$, Figure 1B). Exploration of female-soiled bedding (Figure 1C) was intense in Days 1 and 2 ($P < 0.001$ in both cases) but declined thereafter to about control levels ($P > 0.05$ for both Days 3 and 4). Finally, the time that females spent investigating the male-soiled bedding (Figure 1D) was significantly higher than the time spent investigating the clean bedding in the control situation throughout the experiment ($P < 0.05$ for every test).

To sum up, females do not explore significantly citralva-odorized bedding but show distinct patterns of exploratory behavior of bedding soiled by different conspecifics. Thus, bedding soiled by castrated males and females were explored transiently (although exploration of female soiled was more

persistent), whereas exploration of male-soiled bedding showed no habituation throughout the experiment.

Experiment 2: analysis of reinforcing properties of attractive pheromones

Females of all 3 groups displayed a balanced exploratory behavior of the cage in the control test ($P > 0.4$ in all cases). In each group, the subgroups with soiled bedding presented in the right- and left-side dish displayed a similar exploration time of that dish ($P > 0.1$ in all cases). Therefore, data from both subgroups were pooled for subsequent analysis.

A repeated measures ANOVA with TEST (control, 2nd, 3rd, and 4th days of training and place preference test) as intrasubject factor and GROUP (female, castrated male, and intact male) as between-subject factor revealed a significant TEST × GROUP interaction ($F_{8,108} = 2.564$, $P = 0.013$), a significant main effect of the TEST ($F_{4,108} = 12.877$, $P < 0.001$), and a marginally significant main effect of the GROUP ($F_{2,27} = 3.243$, $P = 0.055$).

Multiple pairwise comparisons of the Female group (Figure 2A) revealed that females significantly explored female-soiled bedding (as compared with the control test) in all the training sessions ($P < 0.002$ in all comparisons) but showed no acquisition of place preference ($P > 0.9$). In the Castrated Male group, there were no significant differences between the control test and any of the different training sessions or the
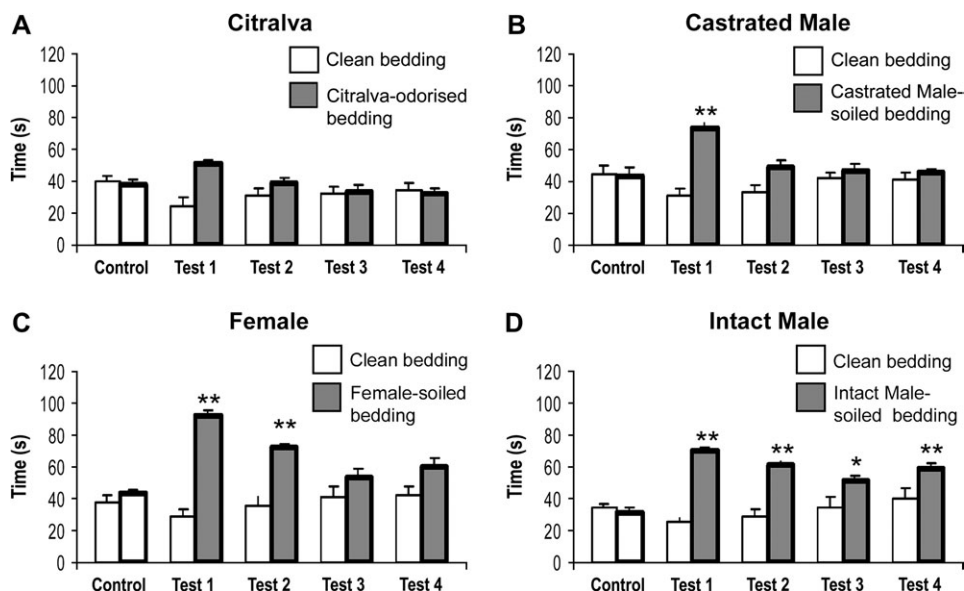
Figure 1 Experiment 1: female mice chemoinvestigatory behavior of conspecific chemosignals and synthetic odorants. The time (mean ± standard error) that females in Experiment 1 spent exploring the dishes that contained citralva-odorized bedding **(A)** and bedding soiled by castrated males **(B)**, intact females **(C)**, or intact males **(D)** in the 4 successive tests (exposures 1–4) is indicated by gray bars. Open bars indicate the exploration time of the dish containing clean bedding in the same tests. In the control situation, in which both dishes contained clean bedding, the bar delineated by thick lines corresponds to the exploration of the dish that will contain the stimulus (citralva or soiled bedding) in exposures 1–4. Asterisks indicate significant differences in pairwise comparisons between the investigation time of a given dish and the time exploring the same dish in the control test (*$P < 0.05$, **$P < 0.01$). Whereas citralva-odorized bedding is not significantly explored (A), bedding soiled by castrated males (B) or adult females (C) elicits a transient, significant chemoinvestigation that habituates by the end of the experiment. In contrast, investigation of male-soiled bedding (D) shows no behavioral habituation through the experiment.
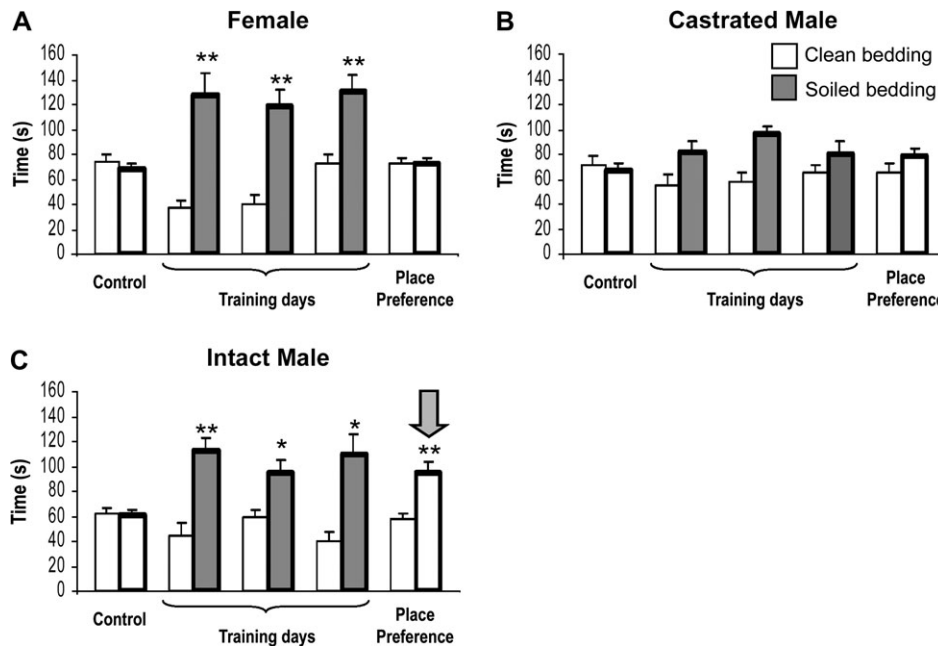
Figure 2 Male sexual pheromones induce place preference on adult female mice. Graphs represent the time (mean ± standard error) spent by the females of groups Female **(A)**, Castrated Male **(B)**, and Male **(C)** on the dish containing soiled (gray bars) or clean bedding (open bars) during training. Like in the control test, in the place preference test clean bedding is located in both dishes (the bars delineated by thick lines in both tests correspond to the exploration of the dish that contains the soiled bedding during training). Asterisks indicate significant differences in pairwise comparisons between the investigation time of a given dish and the time exploring the same dish in the control test ($*P < 0.05$, $**P < 0.01$). Only male-soiled bedding induces a conditioned place preference for the dish where it was previously located (arrow).

place preference test ($P > 0.15$ in all comparisons, Figure 2B). Finally, in the Intact Male group (Figure 2C), every training session significantly differed from the control test ($P < 0.05$). More importantly, the behavior during the place preference test was different from the control test ($P < 0.01$) and similar to the training sessions ($P > 0.9$ in all cases). Thus, during the exposure days (training sessions), females associated male-derived chemical signals with the location in which they were placed and developed a conditioned preference for that location, that is, in the place preference test the place preference was expressed in the absence of the stimulus.

Experiment 3: temporal dynamics of the place preference induced by male chemosignals

Data from the control test revealed no side preference ($t = 1.702$, $P = 0.109$). In addition, the time spent exploring the male-soiled bedding did not differ whether it was located in right- or left-side dishes (Student's t-test, data grouped from all exposure days; $t = 1.58$, $P = 0.12$). Therefore, data from both subgroups were pooled for further analysis.

An ANOVA for repeated measures with TEST as intrasubject factor (control, place preference test, and 3 C/C tests) showed a significant effect of the TEST ($F_{4,60} = 11.037$, $P = 0.001$). Subsequent pairwise comparisons indicated that female mice developed a place preference (Day 5 differed

from control; $P = 0.027$), which rapidly disappeared (no other day differed from control; $P > 0.05$ in all cases, Figure 3). This indicates that, in the absence of the rewarding stimulus (male-soiled bedding), the learned preference for the location decreases quickly.

Experiment 4: analysis of the volatility of the reinforcing male chemical signals

Females of both V and V + NV groups displayed a balanced exploratory behavior of the cage in the control test ($P > 0.2$ in all cases). In both groups, the time spent exploring the male-derived chemicals did not differ whether it was located in right- or left-side dishes (one-way ANOVA, data grouped from all exposure days; in both groups $F < 1$, $P > 0.5$). Therefore, data from both subgroups were pooled for subsequent analysis.

The patterns of the chemoinvestigation of either volatiles or volatiles + involatiles of bedding soiled by intact males versus castrated males were compared by means of a repeated measures ANOVA, with TEST (control and 1st, 2nd, 3rd, and 4th days of training) and STIMULUS (castrated male and intact male) as intrasubject factor and GROUP (V and V + NV) as between-subject factor. This analysis revealed a significant GROUP × STIMULUS interaction ($F_{4,11} = 9.202$, $P = 0.009$), as well as significant main effects of STIMULUS

$(F_{1,14} = 10.82, P = 0.005)$, TEST $(F_{4,11} = 3.837, P = 0.008)$, and GROUP $(F_{1,14} = 11.232, P = 0.005)$. The remaining results of the ANOVA were not significant (P values > 0.1).

The GROUP × STIMULUS interaction indicates a differential preference of both groups for one of the stimuli. Pairwise comparisons show that the V + NV group displayed a preference for male-soiled bedding versus castrated male–soiled bedding (Figure 4A, **P < 0.001), whereas the V group did not (Figure 4B, P = 0.867). Consistent with these results, the analysis of simple effects showed a significant effect of the TEST on the exploratory behavior in the V + NV group (P = 0.004) but no effect (P = 0.315) in the V group. In other words, when contact with the bedding was

allowed, so that the animals could detect volatile and involatile substances, female mice preferred the bedding soiled by intact males to that of castrated males. In contrast, if access to the nonvolatile component of the bedding was prevented, the females showed no preference for either stimulus. Because in the latter situation females presumably are able to detect volatiles emanating from the bedding, it can be concluded that females are attracted to some nonvolatile male chemical signals that are produced in a testosterone-dependent manner. The pattern of this attraction in the V + NV group indicated no habituation throughout the tests. On the other hand, repeated exposure to the volatiles in the V group did not change the exploratory behavior of the animals.

The induction of conditioned place preference by volatile or involatile male chemical signals was analyzed by means of a repeated measures ANOVA with TEST (control and place preference test) and STIMULUS (castrated male–soiled bedding and intact male–soiled bedding during the training sessions) as intrasubject factors and GROUP (V and V + NV) as between-subject factor. This analysis revealed a significant third-order interaction (TEST × GROUP × STIMULUS, $F_{1,14} = 5.714, P = 0.031$), significant GROUP × STIMULUS ($F_{1,14} = 7.904, P = 0.014$) and TEST × STIMULUS ($F_{1,14} = 0.021, P = 0.021$) interactions, and a significant main effect of STIMULUS ($F_{1,14} = 7.582, P = 0.016$). The remaining results of the ANOVA were not significant. As it can be observed in Figure 4 and confirmed by pairwise comparisons, this indicates that conditioned place preference is significantly induced in the V + NV group (control vs. place preference test, **P < 0.001 in Figure 4A) but not in the V group.

In conclusion, this experiment indicates that the signals contained in male-soiled bedding that induced chemoinvestigation and place preference in female mice are nonvolatile
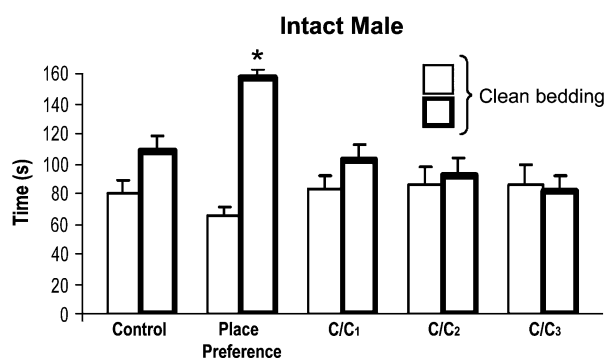

Figure 3   The conditioned place preference induced by male sexual pheromones decreases quickly in the absence of the pheromone. The graph shows the time that the females of Experiment 3 spent exploring both dishes in the control test (Day 0), the place preference test (Day 5), and 3 additional C/C tests (Days 8, 11, and 14). The bars delineated with a thick line correspond to the exploration time of those dishes that contained male-soiled bedding during training sessions (not shown). The asterisk indicates a significant difference in the exploration of a given dish and exploration of the same dish in the control test (P < 0.05 in pairwise comparisons).
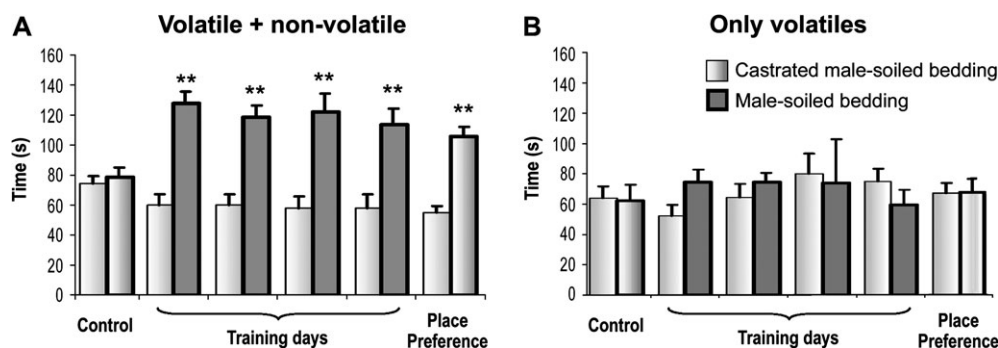

Figure 4   The male sexual pheromone attractive and reinforcing to female mice is involatile. Bars indicate the time that the females of Experiment 4 spent exploring each dish in the control test (Day 0), the place preference test (Day 5), and the 4 training tests, in which they had to choose between bedding soiled by castrated males (light gray bars) and bedding soiled by intact adult males (dark gray bars). In both the control and place preference tests, castrated male–soiled bedding was present in both dishes. (A) The females were allowed to contact the bedding during exploration, thus having access to all of its chemicals (volatile and nonvolatile). (B) Another group of females could not contact the bedding but could detect the volatiles emanating from it through a perforated cover. Statistical analysis (see text) indicates an effect of the group demonstrating that the attractive (significant exploration of male-soiled bedding during training sessions as compared with castrated male–soiled bedding) and reinforcing (significant differences between the place preference test and the control) properties of the bedding are due to nonvolatile, testosterone-dependent, male chemical signals. Double asterisks indicate significant (P < 0.01) differences in pairwise comparisons (see text).

and testosterone dependent. Therefore, some nonvolatile, testosterone-dependent male chemical signals are reinforcing to female mice.

Discussion

The results of Experiment 1 indicate that female mice without previous experience with male-derived chemicals explored significantly more the bedding soiled by other females, castrated males, and intact adult males than clean bedding. Several lines of evidence indicate that this behavior is not just due to novelty effect (e.g., investigation of any new stimulus irrespective of its relevance) but very likely reflects the biological significance of conspecific chemical signals.

We chose citralva as a control for our experiments because it is a synthetic odorant not involved in intraspecific communication, and it had been previously used in habituation–dishabituation tests (Sundberg et al. 1982; Baum and Keverne 2002) to check the olfactory function of mice (Luo et al. 2002; Agustin MC, our unpublished results). In these habituation–dishabituation tests, mice actively explore citralva in the first 1-min presentation, but its chemoinvestigation decreases very quickly. This is compatible with a pure novelty effect and indicates that mice do not find citralva aversive (Luo et al. 2002). In our experiments, citralva-scented bedding shows a faint lemon-like odor to the human nose, so that we assume it is detectable to mice. In spite of it, female mice investigate citralva-odorized bedding just as much as clean bedding (see Results). Therefore, we conclude that our 5-min tests are not suitable to reveal a novelty effect. Even so, in our tests, females significantly investigated bedding soiled by all kinds of conspecifics. Additional evidence that the exploration of conspecifics' chemical signals is not due to a mere novelty effect derives from the intense chemoinvestigation of female-soiled bedding, with which females had previous experience. In addition, chemoinvestigation of the bedding soiled by females and intact males was expressed not only in the first test (when they might constitute a novel stimulus) but also in subsequent ones, when the stimuli were not novel any more.

Therefore, it can be assumed that chemoinvestigation of the chemical signals from conspecifics is due to something else than novelty. This is true even in the case of those derived from castrated males. A possible explanation for this behavior would be that, although bedding soiled by castrated males does not contain chemicals that signal gender or social status (which in rodent males is correlated with testosterone levels; Albers et al. 2002), it includes substances signaling identity and/or genetic profile (individuality), thus being biologically meaningful. In this context, major histocompatibility complex–associated odors and/or peptides, which seem not regulated by testosterone (Peele et al. 2003), have been shown in mice to participate in signaling kin (Manning et al. 1992; Yamazaki et al. 2000) or individual recognition (Leinders-Zufall et al. 2004).

On the other hand, on successive presentation days, only bedding soiled by other females or intact males elicited a persistent investigation. Specifically, our results show that female mice intensely explore (as much as male-soiled bedding; see Figure 1) signals from other females on the first 2 test days. This contrasts to findings by Drickamer (1989) who, using wild stock house mice, showed that adult females avoided other females' chemical cues (contained in their urine or soiled bedding) and reliably preferred water, clean bedding, or just an empty box to female-derived chemical cues. This difference probably reflects the strong territorial behavior of the females used in Drickamer's study (which were third- to fourth-generation descendents of animals trapped in the wild) as compared with outbred animals such as the CD-1 strain used in our study. In any case, both Drickamer's findings and our results coincide in showing a different pattern of exploratory behavior of female mice toward male- and female-derived chemical cues. Thus, in our Experiment 1, females persistently investigated male-derived cues (with no habituation throughout the experiment), but exploration of female-soiled bedding underwent a slow habituation (Figure 1). More importantly, unlike male-derived chemicals, female odorants/pheromones are not able to induce place preference (Figure 2). Therefore, although female-derived chemical signals are not rewarding or reinforcing to other females, our results are consistent with previous observations showing that female-derived chemicals are involved in communication between females (Hurst 1990) because female-soiled bedding elicits intense exploration, a behavioral response that habituates slowly.

VNO-detected male sexual pheromones are rewarding to female mice

The difference between the behavioral responses of females to bedding soiled by intact and by castrated males in Experiments 2–4 indicates that the testosterone-dependent male chemical signals contained in male-soiled bedding are reinforcing to female mice. We assume that these substances are involved in intersexual communication because they elicit reliably responses (attraction and reinforcement) that have a strong influence on the behavior of adult females (see Discussion below). Therefore they fulfil the defining features of sexual pheromones. As far as we know, this finding provides the first experimental demonstration of the unconditioned (nonlearned) reinforcing properties of male sexual pheromones to females in a mammalian species.

In view of this finding, one would expect that females spent significantly more time investigating bedding soiled by adult males than bedding soiled by other kinds of conspecifics (females or castrated males). Therefore, it is surprising that in the first test day of our Experiment 1 (see Results, Figure 1), no significant differences were found in the time females spent investigating male-derived as compared with female-derived or castrated male–derived chemicals. A possible explanation would be that, in a relatively new environment

such as the test cage, the exploratory behavior of the only relevant stimulus (the other dish always contained clean bedding) reaches a ceiling, so that our experimental design was not appropriate to test differences among groups during first test day (when behavioral habituation has not yet occurred). The differences among groups, however, appeared readily in subsequent exposures (Days 2–4 in Experiment 1), as well as in tests that included a learned operant response (Experiment 2).

Two-choice tests are a more suitable experimental design to test differences in the attractiveness of bedding soiled by different conspecifics. Such experiments have been done in other laboratories using different experimental designs (Novotny et al. 1985; Ninomiya and Kimura 1988; Drickamer 1989; Keller, Pierman, et al. 2006), and their results indicate that adult female mice reliably prefer male-derived to female-derived or castrated male–derived chemical stimuli. In our laboratory, we have demonstrated that adult, virgin females that were reared in absence of male-derived secretions/excretions display preference of male-derived over female-derived chemical signals (Moncho-Bogani et al. 2002) and over chemicals derived from castrated males (results of Experiment 4 in the present work). This indicates that this preference is not learned, that is, it has not been acquired as a consequence of previous sexual or "chemosensory" experience but constitutes an unconditioned behavioral response. In addition, it is quite a robust response as it is shown independently of the steroid levels of the females (Moncho-Bogani et al. 2004).

The results of Experiment 4 strongly suggest that reinforcing sexual pheromones are detected by the VNO. In fact, both the unconditioned preference that females exhibit for male sexual pheromones and the conditioned place preference that females develop when rewarded with male-soiled bedding (Figure 4) require of a close contact with the source of the stimulus (bedding), indicating that the chemical signals include nonvolatile components. In this respect, it has been shown in several rodents that the VNO detects under physiological conditions nonvolatile substances (Wysocki et al. 1980; Luo et al. 2003; Moncho-Bogani et al. 2005). In agreement with this, (Keller, Douhard, et al. 2006) showed that the preferred exploration of male-derived versus female-derived or castrated male–derived involatile compounds (mediated by direct nasal contact with the stimulus) is critically dependent of the integrity of the VNO. Although small (volatile) molecules can induce responses in vomeronasal neurons in vitro, it seems that behavioral (this work) or electrophysiological responses in vivo (Luo et al. 2003) require large, involatile molecules acting cooperatively with the volatile molecules (maybe as carriers; Baxi et al. 2006; Keller, Douhard, et al. 2006).

Previous studies by Beauchamp et al. (1983) in guinea pigs indicate that females produce sexual nonvolatile pheromones that are intensely explored by males. In this species, lesions of the VNO of males result in an extinction-like decrease of their chemoinvestigation of female urine (Beauchamp et al. 1982, 1983, 1985). On the basis of this evidence, the authors conclude that VNO-detected female sexual pheromones are primarily reinforcing to male guinea pigs, whereas odorants (detected by the olfactory system) might become secondary reinforcers after learning. Similar roles of the main and accessory olfactory systems in attraction to male sexual pheromones have been suggested in female mice (Moncho-Bogani et al. 2002, 2005).

Role of the main olfactory system in intersexual attraction

Keller, Douhard, et al. (2006) showed that virgin female mice display preference for male-derived volatiles emanating from urine or an anesthetised male as opposed to female- or castrated male–derived volatiles—a preference that is not abolished by VNO lesions but is dependent on the integrity of the olfactory epithelium (Keller, Pierman, et al. 2006). On the other hand, Lin et al. (2005) demonstrated that a single compound present in the urine of intact male mice, (methylthio)-methanethiol (MTMT), which is detected in a specific way in some mitral cells of the main olfactory bulb, partially restores the attractiveness of castrated male urine. Taken together, these data suggest that some airborne odorants detected by the olfactory epithelium, such as MTMT, might be involved in intersexual attraction.

These findings apparently contradict our results showing that females are not attracted to the volatiles emanating from the male-soiled bedding when confronted with those emanating from castrated male– (Experiment 4) or female-soiled bedding (Moncho-Bogani et al. 2002). A possible factor explaining these apparently contradictory results is the previous experience of the animals. The females used in our work and by Moncho-Bogani et al. (2002) were reared in the absence of adult males or their chemical signals. In contrast, the females employed by Lin et al. (2005) were not virgin, so that attraction to MTMT could be secondary to sexual experience—in fact, female mice preferred MTMT when dissolved in castrated male urine but not when dissolved in water, although the mitral cells did not discriminate between these 2 stimuli (Lin et al. 2005). On the other hand, although the female mice used by Keller, Pierman, et al. (2006) were not exposed in the laboratory to any male-derived odors, except when tested, they might have been exposed to adult males before puberty, as it is a common practice to keep the mother and the stud male together until weaning. This possibility is supported by the findings by Moncho-Bogani et al. (2002) who demonstrated that adult females acquire preference for male-derived volatiles after being exposed repeatedly to male-soiled bedding and interpreted this as a Pavlovian conditioned preference to the volatiles. Prepubertal experience might have similar effects on attraction to volatiles, as it has been demonstrated for preputial secretions in female mice (Hayashi 1985) and for synthetic odorants in male rats (Fillion and Blass 1986).

These observations have important implications for the study of the relative importance of the vomeronasal and

olfactory systems in chemical communication in rodents. Both mating and experience with chemical signals of adult conspecifics of the other sex might endow volatiles (airborne odorants) with attractive properties that may mask the effects of experimental manipulation of the chemosensory systems.

Reinforcing sexual pheromones and sexual behavior

Our findings strongly suggest that, in mice, natural rewards include not only sex (mating-elicited somatosensory stimulation), food, water, and sweet taste (Kelley and Berridge 2002) but also some VNO-detected sexual pheromones. The reinforcing properties of sexual pheromones raise the possibility that, at least in rodents, part of the reward derived from sexual activity (in both males and females, see Martinez and Paredes 2001) is due to pheromone detection occurring during mating (precopulatory and copulatory behavior). Consistent with this hypothesis, the integrity of the VNO is essential to maintain the standard rate of mounting behavior in sexually inexperienced male hamsters (Meredith 1986). In addition, male mice lacking certain vomeronasal receptor genes show an apparently normal rate of mounting activity (as compared with wild-type animals) in their first sexual encounters, but their copulatory behavior decays with experience, in contrast to wild-type mice in which there is an experience-dependent increase in sexual activity (Del Punta et al. 2002). Although this deficit might partially be overcome by substantial sexual experience (see Pankevich et al. 2004), the results by Del Punta et al. (2002) suggest that in sexually inexperienced male mice some VNO-detected stimuli (very likely sexual pheromones of the mate) constitute reinforcing cues important to sustain (and increase) the original levels of sexual activity. Our results indicate that a similar situation might be found in sexually inexperienced female mice. In agreement with this, it has been shown that lesion of the VNO in female mice significantly reduce sexual receptivity (Keller, Pierman, et al. 2006).

A recent work of Pankevich et al. (2006) attempted to clarify the role of VNO for the rewarding properties of sex-related stimuli. Their results showed that both VNO-lesioned and VNO-intact male mice acquired place preference for a chamber of the test cage where they could freely explore (but not mount) an anesthetised estrous female. As the authors suggest, this indicates that olfactory stimuli, or even visual or tactile stimuli, derived from the estrous female are rewarding to the male. Our findings and those by Moncho-Bogani et al. (2002, 2005) suggest that in the experiments by Pankevich et al. (2006), female-derived odorants that had been associated to the nonvolatile, primary rewarding sexual pheromones act as secondary reinforcers thus leading to the acquisition of place preference. In fact, Pankevich et al. (2006) gave mating experience to the experimental male subjects in order to maximize the appetitive value assigned to the anesthetised female used as rewarding stimulus to induce place preference. During the sexual encounter, an associa-tion of odorants with rewarding pheromones and rewarding genital stimulation might have occurred.

In agreement with this interpretation, in experienced male rodents, nonvomeronasal stimuli (very likely olfactory ones) are able to sustain sexual behavior in the absence of a functional VNO (Beauchamp et al. 1982, 1985). In fact, the effects of VNO lesions on sexual behavior are much less dramatic in sexually experienced than in inexperienced animals (Meredith 1986). Using a combination of behavioral analysis and Fos expression, Moncho-Bogani et al. (2002, 2005) demonstrated that male-derived olfactory-detected volatiles become attractive to females because of repeated exposure to male-soiled bedding, probably due to classical conditioning. As a consequence, volatiles (olfactory stimuli) become secondary (conditioned) stimuli that would be able to sustain sexual behavior on their own.

Thus, both chemosensory systems would work in tandem in the control of sexual (and probably other) behaviors. The VNO detects (among other signals) nonvolatile pheromones endowed with an intrinsic biological value, thus allowing unconditioned behavioral and physiological responses. In contrast, the main olfactory system detects thousands of airborne odorants, most of which are devoid of intrinsic biological significance. The association of vomeronasal and olfactory stimuli, probably occurring in the basolateral amygdala (Moncho-Bogani et al. 2005), would allow anticipatory conditioned responses to many volatiles (e.g., tracking mates), thus increasing reproductive success. This would explain why a functional olfactory epithelium is needed for the expression of normal levels of sexual behavior (Mandiyan et al. 2005; Keller, Pierman, et al. 2006).

Acknowledgements

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