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Attraction to sexual pheromones and associated odorants in female mice involves activation of the reward system and basolateral amygdala

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Abstract

Adult female mice are innately attracted to non-volatile pheromones contained in male-soiled bedding. In contrast, male-derived volatiles become attractive if associated with non-volatile attractive pheromones, which act as unconditioned stimulus in a case of Pavlovian associative learning. In this work, we study the chemoinvestigatory behaviour of female mice towards volatile and non-volatile chemicals contained in male-soiled bedding, in combination with the analysis of c-fos expression induced by such a behaviour to clarify: (i) which chemosensory systems are involved in the detection of the primary attractive non-volatile pheromone and of the secondarily attractive volatiles; (ii) where in the brain male-derived non-volatile and volatile stimuli are associated to induce conditioned attraction for the latter; and (iii) whether investigation of these stimuli activates the cerebral reward system (mesocorticolimbic system including the prefrontal cortex and amygdala), which would support the view that sexual pheromones are reinforcing. The results indicate that non-volatile pheromones stimulate the vomeronasal system, whereas air-borne volatiles activate only the olfactory system. Thus, the acquired preference for male-derived volatiles reveals an olfactory-vomeronasal associative learning. Moreover, the reward system is differentially activated by the primary pheromones and secondarily attractive odorants. Exploring the primary attractive pheromone activates the basolateral amygdala and the shell of nucleus accumbens but neither the ventral tegmental area nor the orbitofrontal cortex. In contrast, exploring the secondarily attractive male-derived odorants involves activation of a circuit that includes the basolateral amygdala, prefrontal cortex and ventral tegmental area. Therefore, the basolateral amygdala stands out as the key centre for vomeronasal-olfactory associative learning.

Introduction

In female mice, male sexual pheromones affect neuroendocrine functions such as puberty acceleration and oestrous induction, and trigger behavioural responses such as attraction (Halpern & Martínez-Marcos, 2003). In this respect, we have previously shown that sexually mature female mice show an unconditioned attraction to non-volatile components of male-soiled bedding but not to male-derived volatiles (Moncho-Bogani *et al*., 2002, 2004). These findings suggest that the vomeronasal organ (VNO) is involved in detecting the attractive male-derived pheromone, as it possesses a pumping system able to suck non-volatile chemicals (Meredith *et al*., 1980; Wysocki *et al*., 1980). However, the implication of the VNO in the detection of the attractive pheromones has not been proven yet.

Although male-derived volatiles do not attract 'chemically naïve' (reared in the absence of adult male-derived chemicals) female mice, they become attractive by association with non-volatile, innately attractive pheromones, as a consequence of repeated exposure to male-soiled bedding (Moncho-Bogani *et al*., 2002). Because *in vitro* studies have demonstrated that VNO cells respond to several volatiles, including putative pheromones (Sam *et al*., 2001; Boschat *et al*., 2002), the acquired attraction for volatiles could be due to vomeronasal sensitization or to association of two vomeronasal stimuli. Alternatively, non-volatile male sexual pheromones, detected by the VNO, may act as unconditioned stimuli to induce a conditioned attraction for volatiles detected by the main olfactory system (Moncho-Bogani *et al*., 2002). This is consistent with a reinforcing role of VNO-detected sexual pheromones (see Halpern, 1987), suggested by the effects of VNO lesions on chemoinvestigatory behaviour in male guinea pigs (Beauchamp *et al*., 1982, 1985) and on mounting behaviour in male mice (Wysocki *et al*., 1986). Nevertheless, attempts to prove the rewarding properties of vomeronasal stimulation in rodents have been inconclusive (Coppola & O'Connell, 1988).

The experiments presented in this work aim to clarify: (i) which chemosensory systems are involved in investigating male-derived volatile and non-volatile chemicals; (ii) what kind of learning underlies the acquired attraction to volatiles and what neural centres are involved; and (iii) whether sexual pheromones (and, secondarily, associated odours) activate the reward system of the brain (Schultz, 2000, 2002). To clarify these issues we have examined the expression of c-fos in the vomeronasal system, several olfactory nuclei and the mesocorticolimbic dopaminergic system (including the prefrontal

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cortex and amygdala; Kelley & Berridge, 2002) in female mice that actively investigate male-derived chemicals in two different experimental conditions. In the first experiment, 'chemically naïve' females were run through a two-choice preference test (male-soiled vs. clean bedding) prior to Fos immunodetection. The second experiment analyses c-fos expression following a preference test in which chemically inexperienced or experienced females choose between volatiles derived from clean or male-soiled bedding.

Taken together, the results show that non-volatile pheromones stimulate the vomeronasal system whereas air-borne volatiles activate only the olfactory system. Moreover, the reward system is differentially activated by primary (non-volatile) pheromones and secondarily attractive volatiles. Finally, the acquired preference for male-derived volatiles likely represents an olfactory-vomeronasal associative learning that involves the basolateral nucleus of the amygdala.

Materials and methods

Animals

The present studies were performed using female mice ($n = 30$) of the CD-1 strain (Harlan, Barcelona, Spain) that were treated throughout according to the EEC guidelines for European Communities Council Directives of 24 November 1986 (86/609/EEC). To ensure that the females used in the experiments had never been exposed to chemical signals from sexually mature males, 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 were kept in the same room until the age of 9 weeks. Then, females were randomly assigned to five groups ($n = 6$ in each one) and housed in individual cages ($25 \times 50 \times 30$ cm), where the preference tests were going to be run. These cages contained two glass plates of dimensions $5 \times 12 \times 12$ cm, located at opposite corners.

Two-choice preference tests

Before starting the experiments, animals were habituated to handling and to the test conditions for 10 min per day for 4 days. Habituation was performed at the same time of the day in which the preference tests were going to be performed (between 9 h and 13 h). Preference tests (based on Mossman & Drickamer, 1996) were run as in Moncho-Bogani *et al*. (2004). Tests were undertaken in the cages where the animals were housed to minimize both manipulation and exposure to external stimuli. For each test, 10 g of either male-soiled bedding or clean bedding was put in each glass plate. The male-soiled bedding was collected from several cages containing two–five adult males for a week. To ensure a homogeneous composition throughout the experiments, male-soiled bedding from several cages was thoroughly mixed and stored in the freezer until the day of the experiment.

Experiment 1 consisted of a test of the preference of the females for male-soiled bedding in an experimental situation in which the females had direct access to the bedding. This included a control group in which both plates contained clean bedding (C/C) and an experimental group in which the animals were presented with clean vs. male-soiled bedding (C/M). Experiment 2 was a test of the preference of the females for the air-borne volatiles emanating from male-soiled bedding. This was achieved by covering the plates that contained bedding (either clean or soiled by males) with a plastic cover with evenly distributed small holes (3 mm in diameter), thus separating the animals from the bedding by about 4.5 cm. This experiment included a control group in which both plates contained clean bedding (only clean bedding volatiles; C/Cv) and two different experimental (clean vs. male-soiled bedding) groups. The 'inexperienced' group was composed of six females that had never been exposed to chemical signals from sexually mature males (male volatiles, inexperienced females; C/Mvi). This allowed us to measure the preference for male-derived volatiles (vs. volatiles emanating from clean bedding) in their first experience with these chemicals. The second experimental group, the 'experienced' group (male volatiles, experienced females; C/Mve) was composed of females that had been previously exposed daily for 10 min during 4 days to male-soiled bedding in a regular housing cage, following a learning protocol described previously (Moncho-Bogani *et al*., 2002). The exposure to male-soiled bedding in the experienced group took place in a room different from the testing room, to avoid any association of male-derived stimuli with the context in which the test was going to be performed. Twenty-four hours after the last exposure to male-soiled bedding the experienced females were run in the preference test for volatiles.

Using a very similar experimental design for the preference tests, we had previously demonstrated that both sides of the cage were equivalent (Moncho-Bogani *et al*., 2002). Therefore in groups C/M, C/Mvi and C/Mve the plate containing male-soiled bedding was systematically located in the left side of the test cage. After introducing the bedding in the plates, the experimenter left the room and the behaviour was videotaped for the first 5 min. A person who was blind to the experimental conditions measured the time the females spent investigating each plate. An animal was considered to be investigating a plate when it was positioned directly on it. Results are presented (Figs 1 and 2) as the average time in seconds spent investigating each one of the plates ± SE.

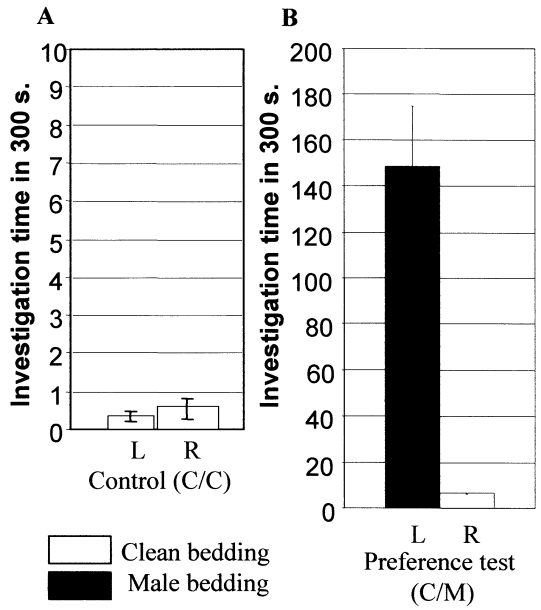


FIG. 1. Females express non-learned preference for male-soiled bedding. Preference test in experiment 1, in which chemically naïve females had direct access to the bedding. (A) Control group, in which females were choosing between two plates, located at opposite corners of the cage (Left, Right), containing clean bedding (clean vs. clean bedding, C/C). (B) Clean vs. male-soiled bedding preference test (C/M). Bars represent investigation time (mean ± SEM) in the 300-s test. As compared with controls, the C/M females showed a clear preference for the plate containing male-soiled bedding. This results in a significant increase in the preference ratio for the male bedding [preference ratio = left/(right + left)] (one-way ANOVA, $P < 0.01$). Accordingly, the preference ratio in the C/M group is significantly higher than 0.5 (Student's *t*-test, $P < 0.001$).

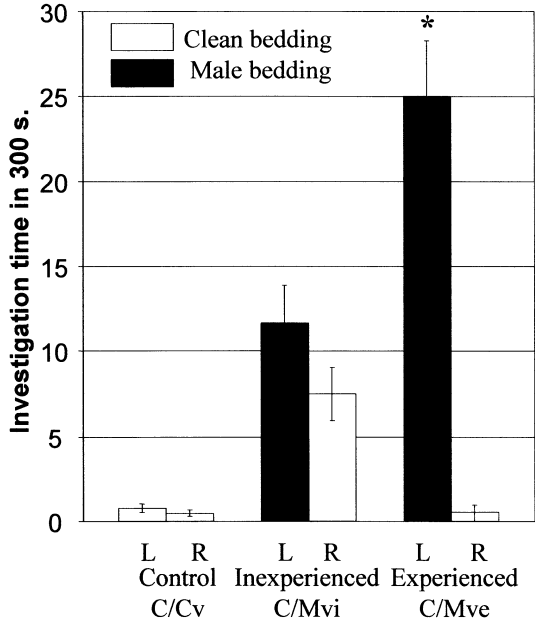


FIG. 2. Experience with male-soiled bedding confers the females attraction to male-derived volatiles. Preference tests in experiment 2, in which chemically naïve females had access only to volatiles derived from the male bedding (see Materials and methods). In the control group (C/Cv) clean bedding was present in both plates. The inexperienced group was composed of chemically naïve females that made a choice between volatiles derived from clean bedding vs. male-derived volatiles located in the left-side plate (C/Mvi). After being exposed repeatedly to male-soiled bedding, the females (experienced group; C/Mve) showed a clear preference for male-derived volatiles. L, left plate; R, right plate. Bars represent investigation time (mean ± SEM) in the 300-s test. An ANOVA analysis of the preference ratios [left/(right + left)] indicates a significant effect of the group ($P < 0.001$), which is due to an increased preference for the male-derived volatiles in the experienced group (*post-hoc* analysis).

Fos immunohistochemistry

The animals were left in the test cages for a total time of 100 min. Then they were deeply anaesthetized using pentobarbital and perfused with saline solution followed by 75 mL of 4% paraformaldehyde in 0.01 M Tris-buffered saline (TBS). Brains were postfixed in the same fixative for 4 h and placed into 30% sucrose–TBS until they sank. The brains were then frozen and 40-µm-thick coronal sections were obtained using a freezing microtome. Free-floating sections were collected in five parallel series, the first one of which was saved in TBS at 4 °C for the immunocytochemical detection of Fos, and the second series was mounted and stained for Nissl staining to help in defining the cytoarchitectonic boundaries of the structures of interest.

For Fos immunocytochemistry, sections were incubated sequentially in: (i) 1% hydrogen peroxide (H_2O_2) in TBS for 30 min at room temperature, for endogenous peroxidase inactivation; (ii) rabbit anti-Fos IgG (Santa Cruz Laboratories, Santa Cruz, CA, USA) diluted 1 : 20,000 in TBS with 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) and 2% normal goat serum (NGS, Vector Laboratories, Burlingame, CA, USA), overnight at 4 °C; (iii) biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted 1 : 200 in TBS with 0.3% Triton X-100 and 2% NGS for 2 h at room temperature; and (iv) avidin–biotin–peroxidase complex (ABC Elite Kit; Vector Laboratories) diluted 1 : 200 in TBS with 0.3% Triton X-100, for 90 min at room temperature. Following each incubation, sections were washed in TBS (3 × 5 min). The resulting peroxidase activity was revealed with 0.025% 3,3′-diaminobenzidine (Sigma) and 0.01 H_2O_2 in TB pH 7.6 for 15 min. Sections were rinsed in 0.2% gelatine in TB and mounted onto slides, dehydrated in alcohols, cleared with xylene and coverslipped with Permount.

Data acquisition and analysis

The structures of interest were identified in the Nissl-stained sections of each animal using the atlas of Paxinos & Franklin (2001). For each structure, the antero-posterior coordinate of the studied section, relative to the interaural line, is provided between parentheses. Analysis of Fos-immunoreactive (Fos-IR) in the vomeronasal system was focused on the anterior (7.36 mm) and posterior (7.0 mm) accessory olfactory bulb, the anterior (2.74 mm), posterodorsal and posteroventral (2.10 mm) divisions of the medial amygdala, the posteromedial cortical amygdala (1.62 mm) and the medial bed nucleus of the stria terminalis (3.94 mm) (von Campenhausen & Mori, 2000). Within the olfactory system the main olfactory bulbs (8.08 mm) and the anterior cortical (2.74 mm) and posterolateral cortical (1.62 mm) amygdaloid nuclei (Scalia & Winans, 1975) were analysed. Finally, the ventral tegmental area (0.72 mm), the nucleus accumbens (shell and core) (4.90 mm), the basolateral complex of the amygdala [anterior (2.74 mm) and posterior (1.62 mm) divisions of the basomedial and basolateral nuclei] and the ventral orbitofrontal cortex (6.26 mm) were analysed to sample the effects of male-derived volatiles and non-volatiles on the reward system (Schultz, 2000, 2002). In the c-fos immunostained series, the indicated levels of these structures were captured with a 5 × objective (except for the main olfactory bulbs, which were captured with a 10 × objective) using a digital camera (Leica DC 300) attached to the microscope. After background subtraction and histogram normalization, the region of interest was selected, using systematic criteria for each centre, as a rectangular or circular counting frame (see Figs 6 and 7). For the main olfactory bulbs the counting frame encompassed the mitral-cell layer and the granular layer, whereas in the accessory olfactory bulbs both layers were analysed separately. Using appropriate image analysis software (Image Processing Tool Kit 4.0, Reindeer Graphics, Asheville, NC, USA; for Adobe Photoshop 5.5) we developed a protocol for automated counting of stained nuclei based on the one described by Wan *et al.* (2001). Briefly, grey-scale images were used and an object (nucleus) was automatically counted when its pixels were >30% grey levels darker than the average grey level of the neuropil of the measured area. This criterion to calculate threshold for binarization was kept constant for all the material. Objects smaller than 30 pixels were discarded (except in the main olfactory bulb, where objects smaller than 15 pixels were discarded). Counts were obtained from rectangular or circular areas (see Figs 6 and 7) and calculated per 0.1 mm^2. Using this method we measured the number of Fos-IR cells in both cerebral hemispheres for each one of the areas of interest, and the density of c-fos-IR cells obtained from both measures was averaged and used for subsequent statistical analyses of c-fos in each structure.

To test this computer-assisted counting method in our material we analysed the number of Fos-IR neurons in the ventral lateral geniculate nucleus in the control and experimental groups of both experiments, where no differences were expected because the lighting conditions were similar for all animals. A one-way ANOVA revealed no differences in Fos expression in this thalamic visual nucleus among the five groups ($F = 0.094$, $P > 0.98$).

Statistics

Behavioural and c-fos expression data derived from both experiments were analysed independently using the SPSS software package (version 11.5).

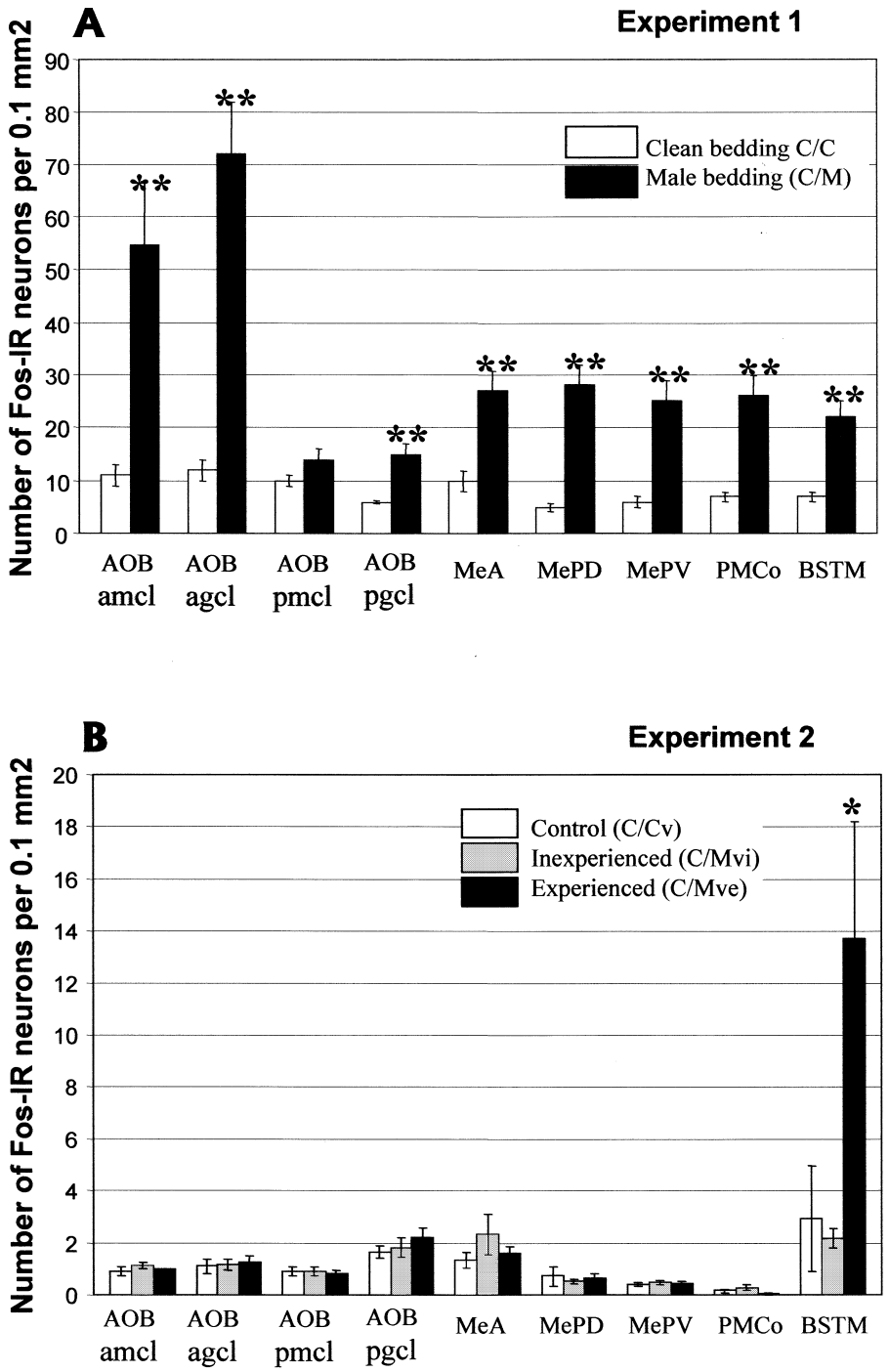


FIG. 3. Exploring male-soiled bedding activates the vomeronasal system of females only when direct access to the bedding is allowed. When chemically naïve females have direct access to the bedding (Fig. 3A, experiment 1), the mean density (± SEM) of Fos-IR neurons (number in 0.1 mm^2) in the vomeronasal system significantly differs between females that explored only clean bedding (C/C, open bars) and those that explored male-soiled bedding (C/M preference test, solid bars). In contrast, male-derived volatiles (Fig. 3B, experiment 2) do not activate the vomeronasal system of females. The density of Fos-IR neurons did not significantly differ in the studied centres of the vomeronasal system in females exposed to volatiles emanating from clean bedding (C/Cv; open bars), in chemically naïve females exposed to male-derived volatiles (C/Mvi; grey bars), which showed no preference for them (see Results) and in females exposed to male-derived volatiles that had previous experience with male-soiled bedding (C/Mve; black bars), which showed attraction to male-derived volatiles (see Results). No effect of the group was observed in any of the studied structures except for the medial bed nucleus of the stria terminalis (BSTM), where *post-hoc* analysis reveals a selective increase of Fos-IR cells in the C/Mve group. This indicates that the BSTM activation is induced by male-derived volatiles only after experience (*$P < 0.05$; **$P < 0.01$). AOB agcl, anterior granular cell layer of the AOB; AOB amcl, anterior mitral cell layer of the AOB; AOB pgcl, posterior granular cell layer of the AOB; AOB pmcl, posterior mitral cell layer of the AOB. For all other abbreviations, see list.

Preference tests

For each test, either in the control (clean- vs. clean-soiled bedding) or in the experimental situation (male- vs. clean-soiled bedding), the ratio between the time spent investigating the left-hand plate and the total time spent investigating the plates was calculated [preference ratio = left/(right + left)]. This preference ratio is about 0.5 when

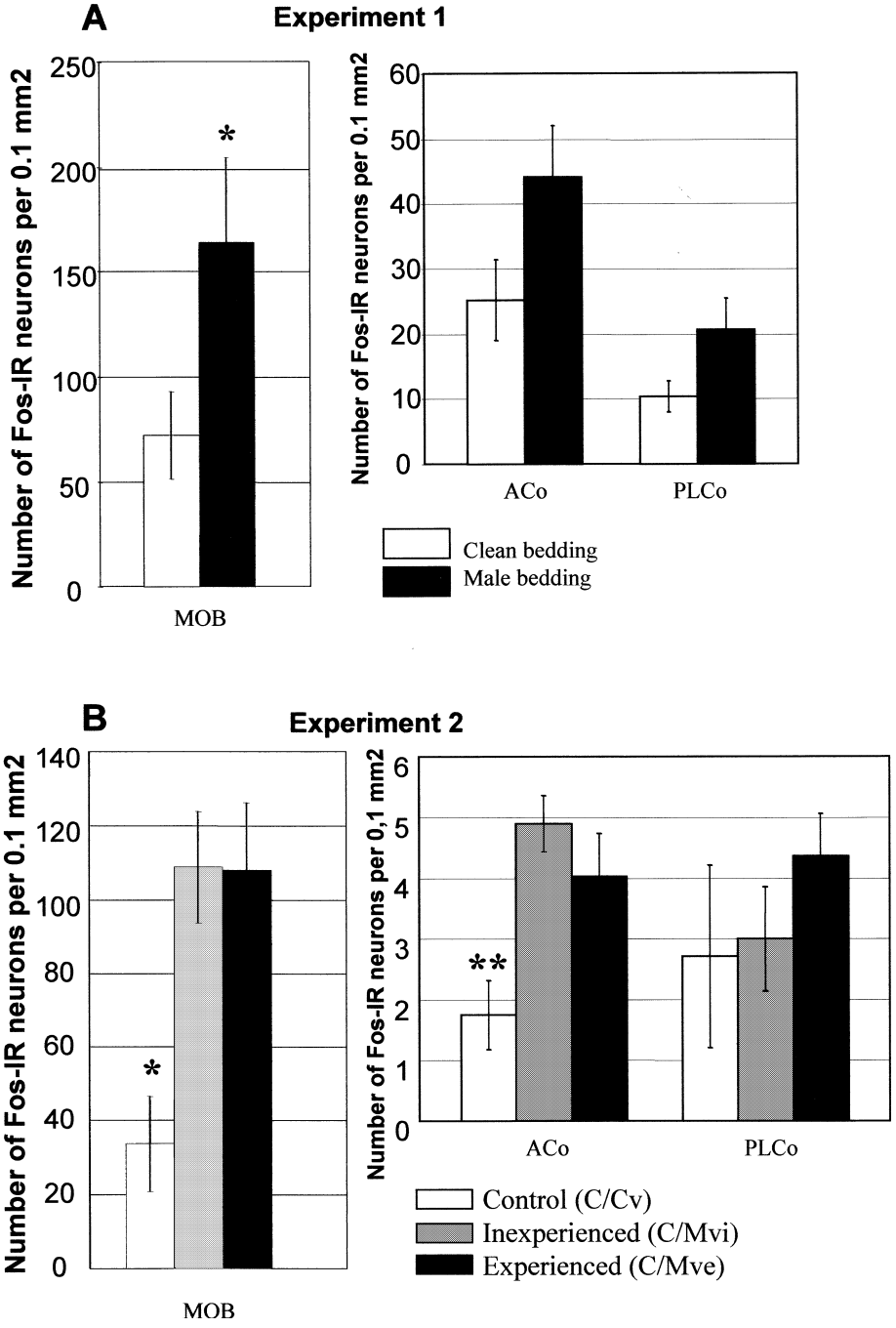


FIG. 4. Exploring directly male-soiled bedding or male-derived volatiles activates parts of the olfactory system of the females irrespective of their previous chemosensory experience. Direct exploration (A, experiment 1) of male-soiled bedding induced a significant increase of the mean density (± SEM) of Fos-IR neurons (number in 0.1 mm^2) only in the main olfactory bulbs ($P < 0.05$) of the chemically naïve females that explored male-soiled bedding (C/M preference test, solid bars) when compared with those that explored clean bedding (C/C, open bars). In experiment 2 (B), when access to non-volatile components of the bedding is prevented, statistical analysis (one-way ANOVA) of the density of Fos-IR neurons in the studied centres of the olfactory system indicates a significant effect of the group (**$P < 0.01$) only in the main olfactory bulb (MOB) and anterior cortical amygdala (ACo). In both centres, a *post-hoc* analysis indicates that this is due to an increment in Fos-IR cells in both the experienced (C/Mve) and inexperienced females (C/Mvi) exploring male-derived volatiles, as compared with females exploring only volatiles emanating from clean bedding (control, C/Cv). For abbreviations, see list.

the female investigates equally both plates, or higher if the animal shows preference for the left-hand plate (containing male-derived pheromones), with a maximum value of 1 when the animal investigates only the left-hand plate. A one-sample *t*-test procedure was used to assess whether the preference ratio in the control situation (both in experiments 1 and 2) is different from 0.5.

Experiment 1. A Kolmogorov–Smirnov test of normality applied to the pooled preference ratios of the preference tests of experiment 1 showed that this parameter follows a normal distribution ($Z = 0.858$, $P > 0.4$). Consequently, a one-way ANOVA was applied to compare the preference ratio in the control and the experimental condition.

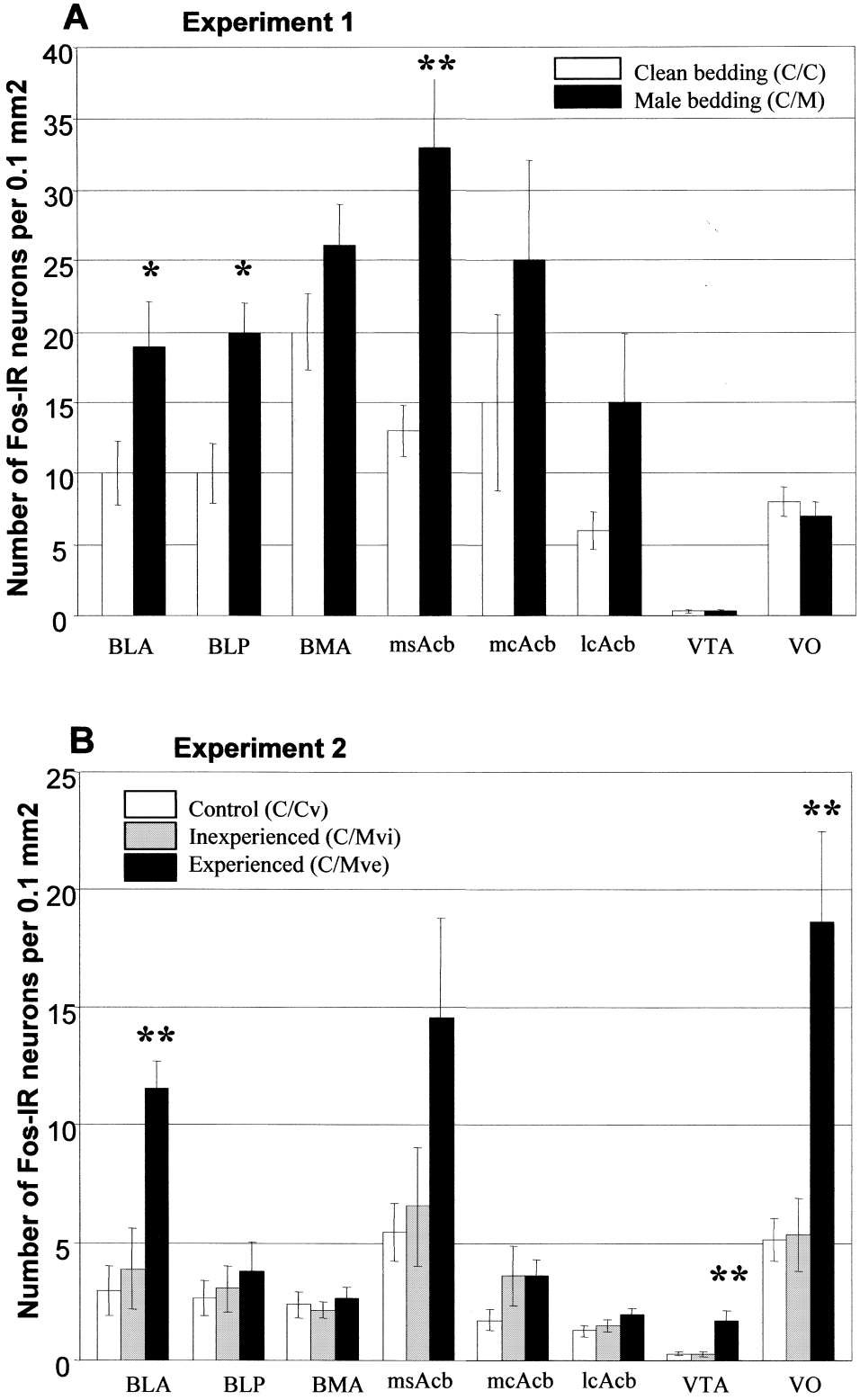
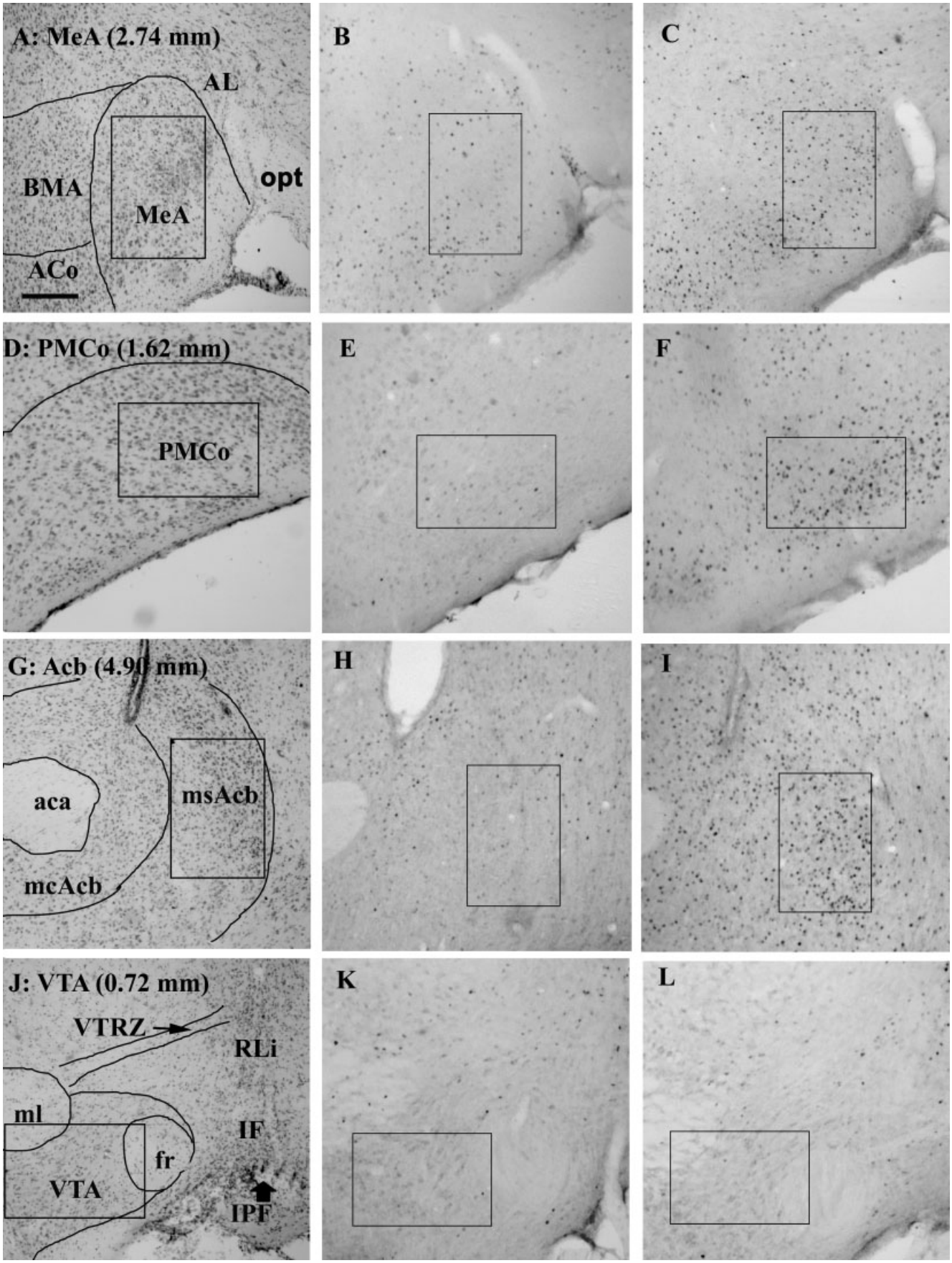


FIG. 5. The brain reward system is differentially activated by the primary pheromones and secondarily attractive odorants. When chemically naïve females had direct access to male-soiled bedding (experiment 1, A) the mean density (± SEM) of Fos-IR neurons (number in 0.1 mm^2) increased in diverse centres of the reward system: a one-way ANOVA revealed a significant activation of the basolateral nucleus of the amygdala and the shell of the accumbens, but not of the remaining structures. In contrast, in experiment 2 (B), where females explore only male-derived volatiles, analysis of the data (ANOVA followed by *post-hoc* analysis) indicates that the density of Fos-IR cells is significantly higher only in the anterior basolateral amygdaloid nucleus (BLA), ventral tegmental area (VTA) and ventral orbitofrontal cortex (VO) of those females having previous experience with male pheromones (C/Mve, solid bars), but not in any of the studied centres of both inexperienced females exploring male-derived volatiles (C/Mvi, grey bars) or females exploring only clean bedding (C/Cv, open bars). IcAcb, lateral core of the Acb; mcAcb, medial core of the Acb; msAcb; medial shell of the Acb. For all other abbreviations, see list. (*$P < 0.05$; **$P < 0.01$).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Experiment 2. Because a Kolmogorov–Smirnov test of normality showed that the preference ratio follows a normal distribution ($Z = 0.706$, $P > 0.7$), a one-way ANOVA was applied to compare the preference ratios in the three groups. A post-hoc Student–Newman–Keuls test allowed further assessment of the differences between groups.

In addition to the preference ratios, in both experiments the total time that the females spent investigating the plates was analysed using a one-way ANOVA.

Fos expression

Experiment 1. To analyse the c-fos induction caused by the investigation of the male-soiled bedding, a one-way ANOVA was applied to compare the mean number of Fos-IR neurons in each one of the measured regions of the female brain in the control vs. the experimental group.

Experiment 2. A one-way ANOVA was applied to analyse the induced c-fos expression in each measured region of the female brain in the three groups. When there was a significant group effect, a post-hoc Student–Newman–Keuls test allowed further assessment of the differences among the groups.

Results

Females show innate attraction for non-volatile pheromones but attraction for volatiles is acquired

In experiment 1, chemically inexperienced females showed no significant preference for any of the plates in the control situation (C/C; preference ratio = 0.56 ± 0.06; Fig. 1A). A one-sample *t*-test revealed that this preference ratio was not significantly different from 0.5 ($t = 0.98$, $P > 0.3$). In contrast, in their first experience with male-derived chemicals (C/M), the females spent significantly more time investigating the plate with male-soiled bedding (preference ratio = 0.95 ± 0.01) (Fig. 1B). The ANOVA comparing the preference ratios in the C/C and C/M groups showed that they are significantly different ($F_{1,11} = 34.9$, $P < 0.001$). In fact, females actively investigate the plate filled with male-soiled bedding for an average time of 148.3 ± 26.6 s, whereas they devote only 6.5 ± 1.2 s to investigate the plate containing clean bedding. In contrast, females showed no interest in investigating any of the plates in the C/C-test. As expected, an ANOVA analysis showed significant differences between the total time that the females of the C/C and C/M groups spent investigating both plates ($F_{1,11} = 34.6$, $P < 0.001$).

In experiment 2 (Fig. 2), chemically inexperienced female mice showed no significant preference for the volatiles emanating from any of the plates in the control situation (C/Cv; preference ratio = 0.61 ± 0.08). A one-sample *t*-test revealed that this preference ratio was not significantly different from 0.5 ($t = 1.35$, $P > 0.2$). The ANOVA comparing the preference ratios in the control (C/Cv), inexperienced (C/Mvi, preference ratio = 0.61 ± 0.02) and experienced (C/Mve, preference ratio = 0.95 ± 0.01) females indicates a significant effect of the group ($F_{2,17} = 13.31$, $P < 0.001$) (Fig. 2). A *post-hoc* Student–Newman–Keuls test applied to explore the differences between groups (with a common level of significance $\alpha = 0.05$) indicates that there are two sets of homogeneous means. There are no significant differences between preference ratios of the C/Cv and the C/Mvi groups, whereas the females of the C/Mve group spent significantly more time exploring volatiles from the plate containing male-soiled bedding. This confirms our previous observations (Moncho-Bogani *et al.*, 2002) in similar two-choice preference tests using male- vs. female-soiled bedding, indicating that some non-volatile components of the male-soiled bedding are attractive to previously inexperienced females, whereas male-derived volatiles do not attract females but acquire attractive properties by their association with the innately attractive non-volatile pheromone(s).

The presence of male-derived volatiles induced a significant increase in the total investigation time of the plates containing the volatile stimuli, as revealed by an ANOVA comparing the total time spent investigating both plates in the three groups ($F_{2,17} = 18.8$, $P < 0.001$). A *post-hoc* Student–Newman–Keuls test indicates that there are no significant differences between the total time that the females of the C/Mve and C/Mvi groups spent investigating both plates, whereas the females of the C/Cv group spent significantly less time ($P < 0.05$) exploring the volatiles originating from the plates.

Non-volatile pheromones stimulate the vomeronasal system whereas volatiles are detected by the olfactory system

The combined study of behaviour (attraction to male-soiled bedding) and the c-fos expression (induced by the exploration of the bedding) in the same animals allows us to analyse the sensory systems that female mice employ in the exploration of chemical signals derived from male conspecifics in each experimental situation. The analysis of the density of Fos-IR cells in each one of the studied structures indicates that, if direct contact with it is allowed, exploration of male-soiled bedding activates the vomeronasal system (Fig. 3A) and, to a lesser degree, also the olfactory system (Fig. 4A), including the olfactory bulbs and some structures of the chemosensory amygdala.

Thus, an ANOVA comparing the density of Fos-IR cells in the C/C and C/M groups of experiment 1 indicates that exposure to male-soiled bedding significantly augmented the number of Fos-IR cells (Fig. 3A) in both the mitral ($F_{1,11} = 11.53$, $P < 0.01$) and granular layers ($F_{1,11} = 33.38$, $P < 0.01$) of the anterior accessory olfactory bulb (AOB). In contrast, the posterior AOB displayed a relatively small increment of Fos-IR cells that was significant for the granular layer ($F_{1,11} = 15.55$, $P < 0.01$) but not for the mitral layer ($F_{1,11} = 3.51$, $P = 0.09$).

Moreover, the vomeronasal amygdala was clearly activated (Fig. 3A) in those animals in which contact with the male-soiled bedding was allowed, as indicated by a significant increment in the density of Fos-IR cells in the anterior (MeA, Fig. 6A–C; $F_{1,11} = 10.82$, $P < 0.01$), posterodorsal (MePD) ($F_{1,11} = 30.87$, $P < 0.01$) and posteroventral portions of the medial amygdala (MePV; $F_{1,11} = 26.31$, $P < 0.01$), and in the posteromedial cortical amygdala (PMCo, Fig. 6D–F; $F_{1,11} = 20.44$, $P < 0.01$). Finally, in the medial division of the bed nucleus of the stria terminalis (BSTM) exposure to male bedding significantly augmented the number of Fos-IR cells ($F_{1,11} = 23.69$, $P < 0.01$).

FIG. 6. Immunoreactivity for c-fos after exploring clean and male-soiled bedding (experiment 1). Digital photomicrographs showing several of the structures of interest in which c-fos expression has been analysed. Nissl-stained adjacent sections, which were used to delineate the cytoarchitectonic boundaries of the structures of interest, are shown on the left column (A, D, G, J), with indication of the antero-posterior level with respect to the interaural line and the main cytoarchitectonic landmarks (Paxinos & Franklin, 2001). The middle column (B, E, H, K) shows photomicrographs of the corresponding regions in c-fos immunostained sections of females of the control group in experiment 1 (C/C). In the right column (C, F, I, L) we show c-fos immunoreacted equivalent sections from females belonging to the group that explored male- vs. clean bedding in experiment 1 (C/M). The structures shown are the anterior medial amygdala (MeA, upper panel, A–C), the posteromedial cortical amygdaloid nucleus (PMCo, second panel, D–F), the medial shell of nucleus accumbens (msAcb, third panel, G–I) and the ventral tegmental area (VTA, lower panel, J–L). Calibration bar in A, valid for the rest of the photographs, equals 100 μm. Rectangles delineate the counting regions. aca, anterior limb of the anterior commissure; AL, nucleus of the ansa lenticularis; fr, fasciculus retroflexus; IF, interfascicular nucleus; IPF, Interpeduncular fossa; mcAcb, medial core of the Acb; ml, medial lemniscus; opt, optic tract; RLi, linear raphe nucleus; VTRZ, visual tegmental relay zone. For all other abbreviations, see list.

In this same experiment, the olfactory system seems partially activated (Fig. 4A). Thus, c-fos expression showed a significant increment in the main olfactory bulb (MOB; $F_{1,11} = 6.12$, $P < 0.05$) of animals exposed to male-soiled bedding (C/M) as compared with controls (C/C), whereas the increment of c-fos was not statistically significant in the two olfactory amygdaloid nuclei analysed, the anterior cortical amygdala (ACo; $F_{1,11} = 3.56$, $P = 0.088$) and the posterolateral cortical amygdala (PLCo; $F_{1,11} = 3.88$, $P = 0.077$).

In experiment 2, where animals had access only to the volatiles emanating from male-soiled bedding through a perforated plastic cover, the analysis of c-fos expression indicates that male-derived volatiles activate the olfactory but not the vomeronasal system. In the olfactory system (Fig. 4B), the ANOVA of the number of Fos-IR cells in the MOB in all three groups of this experiment (control, C/Cv; preference for male volatiles in inexperienced females, C/Mvi; preference for male volatiles in experienced females, C/Mve) indicated an effect of the group on the c-fos expression ($F_{2,17} = 6.98$, $P < 0.01$). The *post-hoc* analysis revealed a significant increment of Fos-IR cells in the C/Mvi and C/Mve groups with respect to the C/Cv ($P < 0.05$), the inexperienced and experienced groups having homogenous means. Consistent with these results, the number of Fos-IR cells in the ACo was dependent on the group ($F_{2,17} = 7.62$, $P < 0.01$), and the *post-hoc* analysis revealed a significant (although small) increment of Fos-IR in the C/Mvi and C/Mve groups as compared with the C/Cv ($P < 0.05$). Surprisingly, the number of Fos-IR cells in the PLCo showed no significant differences among groups ($F_{2,17} = 0.66$, $P > 0.5$).

In contrast to the olfactory system, none of the studied centres of the vomeronasal system showed activation by male-derived volatiles (Fig. 3B), with the exception of the BSTM (see below). Thus, the ANOVA analysis indicates that neither in the anterior AOB (mitral layer: $F_{2,17} = 0.35$, $P > 0.7$; granular layer: $F_{2,17} = 0.18$, $P > 0.8$) nor in the posterior AOB (mitral layer: $F_{2,17} = 0.59$, $P > 0.5$; granular layer: $F_{2,17} = 0.10$, $P > 0.8$) the group had a significant effect on the number of Fos-IR cells. A similar situation is found in the medial amygdala (MeA: $F_{2,17} = 1.05$, $P > 0.3$; MePD: $F_{2,17} = 0.20$, $P > 0.8$; MePV: $F_{2,17} = 0.17$, $P > 0.8$) and PMCo ($F_{2,17} = 0.41$, $P > 0.6$). In contrast, for the other secondary vomeronasal centre, the BSTM (Fig. 7G–I), a significant effect of the group on the number of Fos-IR cells was found ($F_{2,17} = 5.08$, $P < 0.05$). Interestingly, the *post-hoc* analysis indicates that the increment in Fos-IR cells is restricted to the C/Mve group ($P < 0.05$). These data suggest that activation of the BSTM by volatiles is induced by previous experience, and that this occurs through some intervening associative centres rather than due to a direct stimulation from the AOB.

Differential activation of the reward system following exposure to primary (non-volatile) pheromones and volatiles with learned attractive properties

We have analysed the expression of c-fos found after active exploration of male-soiled bedding in both experimental conditions (experiment 1: direct access to the bedding; experiment 2: access to volatiles) in the key centres of the reward system, namely the basolateral division of the amygdala, the ventral tegmental area, the shell and core of the nucleus accumbens and the orbitofrontal cortex. Within the basolateral division of the amygdala, the lateral nucleus and the posterior portion of the basomedial nucleus consistently displayed extremely low numbers of Fos-IR reactive cells. Therefore we restricted our analysis to the anterior and posterior portions of the basolateral nucleus and the anterior basomedial nucleus.

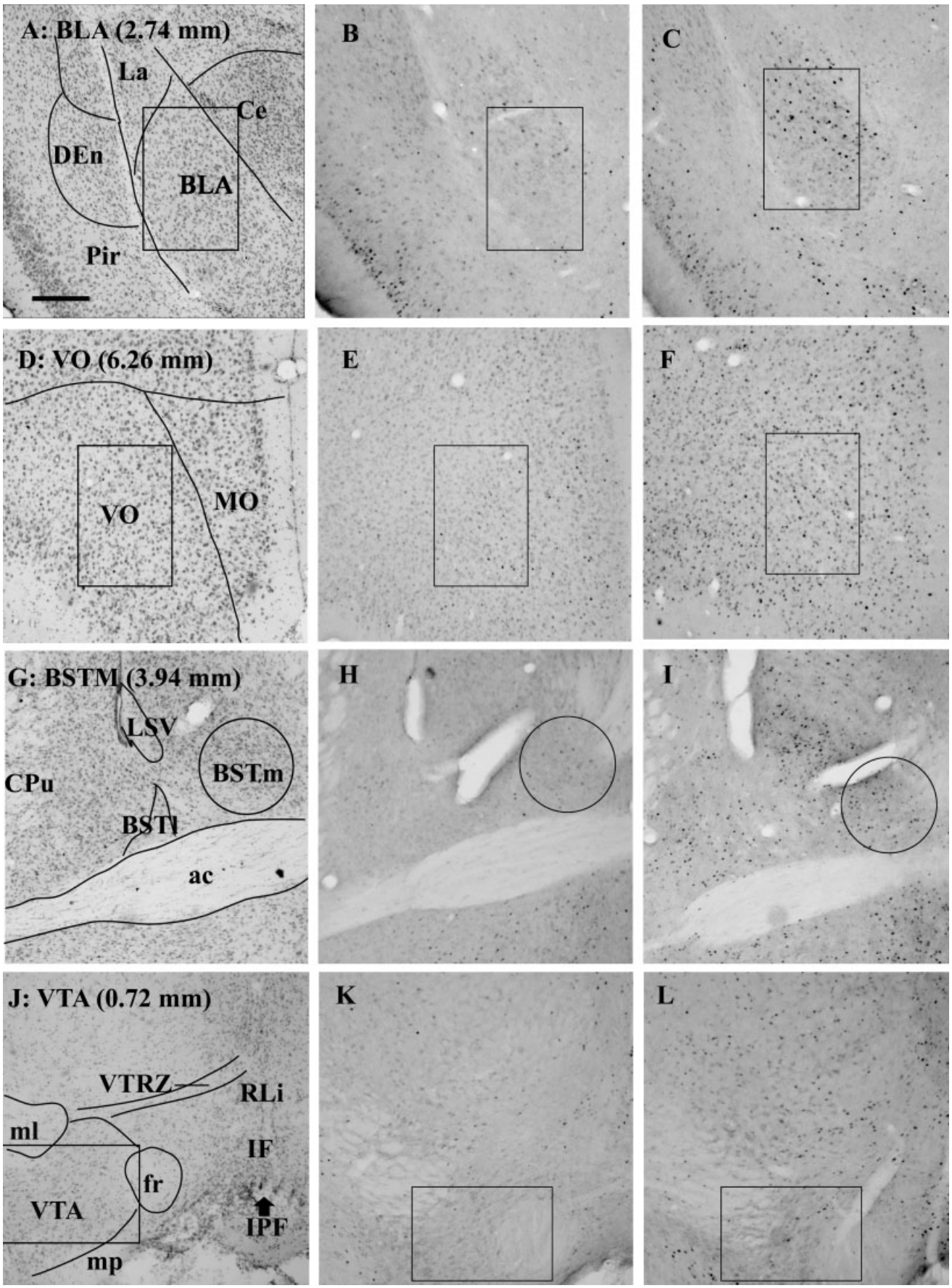
In experiment 1 (Fig. 5A), comparison of the number of Fos-IR cells between the C/C and C/M groups revealed a significant activation of both the anterior (BLA: $F_{1,11} = 5.64$, $P < 0.05$) and posterior (BLP: $F_{1,11} = 6.64$, $P < 0.05$) portions of the basolateral amygdaloid nucleus, whereas no significant differences were found in the anterior basomedial nucleus (BMA: $F_{1,11} = 2.36$, $P > 0.15$). The nucleus accumbens (Acb) was heterogeneous concerning its response to exploration of male-soiled bedding. Thus, a significant activation of c-fos expression was found in the medial shell of the Acb (Fig. 6G–I; $F_{1,11} = 15.24$, $P < 0.01$), whereas neither the medial ($F_{1,11} = 0.98$, $P > 0.34$) nor the lateral core ($F_{1,11} = 2.58$, $P > 0.13$) displayed a significant induction of c-fos expression. The ventral orbital cortex (VO) showed no significant increment of c-fos expression ($F = 0.06$, $P > 0.8$). Finally, it is interesting to note that the ventral tegmental area (VTA) of both control animals and animals that had explored male-soiled bedding was virtually devoid of Fos-IR cells (Fig. 6J–L), and no differences were found between the C/C and C/M groups ($F_{1,11} = 0.06$, $P > 0.80$).

In experiment 2, following the exploration of volatiles emanating from male bedding (Fig. 5B), within the basolateral amygdaloid division the ANOVA revealed an effect of the group in the number of Fos-IR cells in the BLA (Fig. 7A–C) ($F_{2,17} = 12.20$, $P < 0.01$) but not in the posterior portion of this nucleus (BLP: $F_{2,17} = 0.36$, $P > 0.7$) or in the BMA ($F_{2,17} = 0.27$, $P > 0.7$). In the BLA the *post-hoc* analysis revealed a significant increment of Fos-IR in the C/Mve group with respect to the C/Mvi and C/Cv groups ($P < 0.01$). In spite of the apparent increase in the number of Fos-IR cells in the medial shell of the Acb (Fig. 5B), this was non-significant as indicated by the ANOVA analysis (no effect of the group; $F_{2,17} = 2.85$, $P = 0.08$). In the core of the Acb, the exploration of volatiles emanating from male bedding had no effect (medial core: $F_{2,17} = 1.55$, $P > 0.2$; lateral core: $F_{2,17} = 2.13$, $P > 0.1$).

In contrast to experiment 1, in experiment 2 the VTA (Fig. 7J–L) showed a certain number of Fos-IR cells. Moreover, the statistical analysis indicated a significant effect of the group on the density of Fos-IR cells in the VTA ($F_{2,17} = 8.89$, $P < 0.01$), and the *post-hoc* analysis revealed that an increment of Fos-IR was present in the C/Mve group ($P < 0.01$). Finally, a significant effect of the group on c-fos expression was also clear for the VO (Fig. 7D–F) ($F_{2,17} = 10.07$, $P < 0.01$), due to an increment of Fos-IR cells in the C/Mve group, as revealed by the *post-hoc* analysis ($P < 0.01$).

These results show an activation of the reward system of the brain of females that display a preference for chemical stimuli derived from male-soiled bedding. However, the pattern of activation of the reward

FIG. 7. Immunoreactivity for c-fos after exploring volatiles derived from male-soiled bedding with and without previous experience (experiment 2). Digital photomicrographs of Nissl-stained sections (left column) and of equivalent Fos-immunostained material from 'chemically naïve' females (C/Mvi; middle column) and from 'chemically experienced' females that had been run in a test for preference between volatiles derived from clean and male-soiled bedding (C/Mve; right column) in experiment 2. The antero-posterior coordinate of the sections relative to interaural line and the main cytoarchitectonic landmarks (Paxinos & Franklin, 2001) are indicated on the Nissl-stained section. The structures shown are the anterior basolateral amygdala (BLA; upper row, A–C), the ventral orbital cortex (VO; second row, D–F), the medial bed nucleus of the stria terminalis (BSTM; third row, G–I) and the ventral tegmental area (VTA; lower row, J–L). Calibration bar in A, valid for the rest of the photographs, equals 100 μm. Delineated areas in the middle and right columns identify the regions where Fos-IR cells were counted. ac, anterior commissure; BSTL, lateral bed nucleus of the stria terminalis; Ce, central amygdala; CPu, caudatus putamen; DEn, dorsal endopiriform nucleus; fr, fasciculus retroflexus; IF, interfascicular nucleus; IPF, interpeduncular fossa; La, lateral amygdala; LSV, lateral septal nucleus, ventral part; ml, medial lemniscus; MO, medial orbital cortex; mp, mammillary peduncle; Pir, piriform cortex; RLi, linear raphe nucleus; VTRZ, visual tegmental relay zone. For all other abbreviations, see list.


A: BLA (2.74 mm)
La
Ce
DEn
BLA
Pir
B
C
D: VO (6.26 mm)
VO
MO
E
F
G: BSTM (3.94 mm)
LSV
CPu
BSTm
BSTl
ac
H
I
J: VTA (0.72 mm)
VTRZ
RLi
ml
IF
fr
VTA
IPF
mp
K
L

system elicited by the exploration of non-volatile, innately attractive pheromones differs from the one induced by the investigation of volatiles that have become attractive by their association with pheromones. Exploration of the innately attractive non-volatile pheromones activates the basolateral nucleus of the amygdala and the shell of nucleus accumbens, whereas the VTA and VO are not activated as compared with controls. In contrast, those animals showing an acquired attraction for volatiles (due to their association with pheromones) display activation of the BLA (but not the BLP), as well as of the VTA and VO, whereas the accumbens (shell or core) is not activated.

Discussion

The vomeronasal system detects pheromones with a non-volatile component

The most likely explanation for our results on c-fos expression is that male-derived volatiles are detected by the olfactory epithelium but do not activate the VNO of females (see experiment 2) whereas, as put forward by Wysocki *et al*. (1980), the vomeronasal system is involved in the detection of male-derived non-volatile complexes (maybe formed by volatiles associated to non-volatiles, Humphries *et al*., 1999; Yamaguchi *et al*., 2000; Hurst *et al*., 2001; Nevison *et al*., 2003; Pankevich *et al*., 2004). In fact, Leinders-Zufall *et al*. (2004) have very recently identified as ligands of the VNO non-volatile peptides associated with the major histocompatibility complex class I molecules. This finding is consistent with the results by Luo *et al*. (2003), who reported that the mitral cells of the AOB are not activated at a distance (as would be expected if volatiles were involved), but only when physical contact with the source of chemical stimuli (anaesthetized conspecifics) occurs.

The only behavioural evidence for a role of the VNO in the detection of volatiles derives from the work of Trinh & Storm (2003), who reported that type-3 adenylyl cyclase knockout mice, whose olfactory epithelium has a non-functional cAMP-signalling pathway, are able to detect several odorants, including putative pheromones (2-heptanone and dimethyl pyrazine). However, Lin *et al*. (2004) have unequivocally demonstrated that mice defective on the olfactory cAMP-signalling pathway detect some odorants (including heptanone and dimethyl pyrazine) through their olfactory epithelium using alternative transduction pathways.

Additional evidence favouring VNO detection of volatiles derives from *in vitro* analysis of the activity of excised VNO neurons indicating that they respond to aqueous solutions of synthetic urine-borne volatiles (Leinders-Zufall *et al*., 2000; Sam *et al*., 2001; Boschat *et al*., 2002; Del Punta *et al*., 2002). Reconciling this with our results and those of Luo *et al*. (2003) is possible by assuming that VNO neurons are sensitive to some urine-borne volatiles through very specific receptors (Boschat *et al*., 2002), but during exploratory behaviour either these volatiles need a carrier protein to reach functional concentrations at the VNO, or the cooperative action of non-volatiles (such as major urinary proteins, see Beynon & Hurst, 2004) is required to activate mitral cells in the accessory olfactory bulb. Convergent projections of VNO cells expressing different V1 receptors onto individual glomeruli (Belluscio *et al*., 1999) might subserve this cooperative action.

Sexual pheromones, related odours and reward

The rewarding properties of vomeronasal stimulation were first suggested by Beauchamp *et al*. (1982, 1985). However, the first attempts to check this hypothesis in rodents using sexual pheromones were inconclusive (Coppola & O'Connell, 1988). Our results confirm and expand previous findings of our group (Moncho-Bogani *et al*., 2002), indicating that non-volatile male-derived chemicals are intrinsically attractive and are able to induce a conditioned attraction to volatiles, probably by means of a Pavlovian-like association in which pheromones would act as unconditioned reinforcing stimuli. Our data on c-fos expression indicate that exploration of non-volatile primarily attractive male sexual pheromones mainly activates the anterior accessory olfactory bulb of females. This suggests a rewarding value of a sexual pheromone detected by type V1 vomeronasal receptors (expressed by sensory neurons projecting to the anterior AOB; Belluscio *et al*., 1999; Rodríguez *et al*., 1999). In agreement with this, male mice having a deletion of 16 genes of the V1ra and V1rb families display decreasing sexual behaviour with repetitive encounters with a female, in contrast to the increase in sexual activity observed in wild-type mice, a result interpreted by the authors as consistent with the rewarding properties of (V1-mediated) VNO stimulation (Del Punta *et al*., 2002).

Our c-fos data indicate that the investigation of male-soiled bedding activates the reward system (experiment 1) in the female brain, including the BLA, BLP and shell of the Acb, but neither the VTA nor the VO. Thus, male-derived chemicals with unconditioned attractive properties (putative rewarding pheromones) apparently do not induce the activation of the tegmento-striatal pathway but of amygdalo-accumbens projections instead (Brog *et al*., 1993; Wright *et al*., 1996). This is consistent with previous studies indicating a role of the amygdala in reward (reviewed by Baxter & Murray, 2002), which is mainly mediated by the amygdaloid projections to the ventral striatum (Everitt & Robbins, 1992). In fact, self-stimulation of the amygdala induces lever pressing at very low intensities as compared with stimulation of the medial forebrain bundle (Kane *et al*., 1991a,b). Therefore, pheromone-induced reward might be a dopamine-independent process (Cannon & Palmiter, 2003), thus contradicting the dopamine hypothesis of reward (Wise, 1996). Alternatively, vomeronasal stimulation by sexual pheromones might elicit VTA-independent dopamine release in the Acb mediated by amygdaloid inputs, as demonstrated using electrical stimulation of the amygdala by Floresco *et al*. (1998).

Experiment 2 suggests that male-derived volatiles become rewarding secondarily due to their association with primary rewarding non-volatile pheromones. Thus, volatiles activate the BLA, VTA and VO only in experienced (C/Mve) females, whereas the Acb shows non-significant c-fos induction. A first conclusion drawn from these results is that primary and secondary rewards use different brain mechanisms and circuits. Although negative results of c-fos expression should be taken with caution (see Kovacs, 1998), our c-fos data fit the view that the mesocorticolimbic dopaminergic systems signal the incentive salience of reward-related cues, and not the rewarding properties themselves (see Kelley & Berridge, 2002). Thus, when exploring pheromones for the first time (experiment 1), females are not pursuing reward-related cues and, accordingly, VTA cells are not activated. In contrast, in experiment 2 odorants are reward-predicting cues with clear incentive salience, which results in activation of VTA neurons of the females that are engaged in a goal (pheromone)-directed behaviour. In view of our c-fos results, prefrontal afferents to the VTA (Phillipson, 1979; our unpublished results in mice) appear as the most likely pathway for VTA activation by volatiles in females having previous experience with male sexual pheromones and their associated volatiles.

Olfactory-vomeronasal convergence: the role of the amygdala in associative learning

Our results confirm that repeated exposure of female mice to bedding soiled by conspecific males renders an acquired preference for

otherwise unattractive male-derived volatiles. Fos expression demonstrates that this is not due to a sensitization of the VNO to volatiles, but it is an acquired attraction for olfactory stimuli (odorants) due to their association to non-volatile VNO-detected pheromones. An alternative explanation for this phenomenon, namely a volatile-to-volatile (odorant-to-odorant) association, is not likely as it would involve the existence of an innately attractive odorant, which is disproved by the lack of preference for the volatiles displayed by inexperienced females (C/Mvi group in experiment 2, see also Moncho-Bogani *et al*., 2002). Finally, we want to point out that the possibility of pseudoconditioning, that is observing an attraction to the volatiles due to a non-associative process (such as a general sensory arousal) induced by exposure to male non-volatiles, cannot be discarded with the present data. However, if the olfactory and/or the vomeronasal systems were sensitized due to the previous experience with male-derived non-volatiles, then the olfactory and/or vomeronasal sensory centres would display an increase in Fos reactivity in the experienced group vs. the inexperienced group in experiment 2, a result that was not observed.

Therefore, the most likely explanation for the preference for male-derived volatiles displayed by experienced females (C/Mve group) is that it is a learned response due to a Pavlovian-like association of an innately attractive VNO-dependent pheromone (unconditioned stimulus) with olfactory-dependent odorants that act as conditioned stimulus, which allows a transference of the attractive properties from the former to the latter.

This association must have occurred in a centre where olfactory and vomeronasal information converges. Vomeronasal and olfactory systems use parallel pathways targeting mainly non-overlapping portions of the amygdala (Halpern, 1987; Keverne, 1999; but see Licht & Meredith, 1987). Nevertheless, intrinsic amygdaloid circuitry (see Pitkänen, 2000) plus projections from the piriform cortex to the amygdala (McDonald, 1998) might allow association of both kinds of stimuli within the basolateral division of the amygdala (our unpublished results in mice). This is supported by our c-fos data indicating that during learning (first exposition of females to pheromones and odorants from male-soiled bedding, experiment 1) the BLA and BLP become activated, and the expression of this memory (experiment 2, C/Mve) induces the activation of the BLA.

Another structure that may play a role in the association of rewarding pheromones with neutral odorants is the VO, as it has been demonstrated that many prefrontal neurons encode a representation of olfactory stimuli that is dependent on their association with a reward (usually taste; see Rolls, 2000). Nevertheless, our c-fos data do not support a primary role of the VO in odour–pheromone association, as the VO is not activated during learning (experiment 1, group C/M) but only during the expression of the acquired preference for volatiles (experiment 2, group C/Mve). In conclusion, our data point to a primary role of the basolateral amygdala in olfactory-vomeronasal association during Pavlovian-like conditioning, and a secondary role for the VO. This fits the results of Schoenbaum *et al*. (1999) who reported that activation of BLA neurons by odorants predicting sweet or bitter tastes preceded the changes in behavioural response to the odour, whereas orbitofrontal neurons responded only when a learned behaviour was established. Therefore, the basolateral amygdala seems to play a general role in assigning an emotional value (thus motivational significance) to incoming stimuli (including odorants), whereas the prefrontal cortex might use this information to generate adaptive behaviours. For instance (see Discussion above), by means of its projection to the VTA the orbitofrontal cortex might induce activation of the reward system by volatiles.

Olfactory and vomeronasal systems: a tandem for the detection of semiochemicals

Our findings suggest an important role of the vomeronasal-olfactory association occurring in the basolateral amygdala. Vomeronasal and olfactory systems are differently designed (see Mombaerts, 2004) to detect different kinds of chemicals from the environment. The olfactory system detects a myriad of air-borne volatiles emanating from distant sources, using generalistic receptors. This unspecific receptor–ligand binding precludes a hard-wired response to odorants. On the contrary, the vomeronasal system seems designed to detect with great specificity complex pheromones (conformed by non-volatile proteins attached to volatile ligands) that elicit non-learned adaptive responses. Moreover, detection of odorants occurs while breathing, whereas the vomeronasal system (like the gustatory system) detects nearby chemicals using active exploratory behaviours (e.g. vomeronasal pumping; Meredith *et al*., 1980).

Pavlovian association between odours, acting as conditioned stimuli, and tastes or VNO-detected semiochemicals acting as unconditioned stimuli (with an intrinsic emotional value), allows emotional tagging of odorants. Being detected at a long distance, emotionally tagged odorants allow anticipatory adaptive responses. Thus, animals can detect in advance and track the odours of possible mates (non-volatile sexual pheromones, this work) or preys (worm-derived glycoproteins detected by the VNO of snakes; Wang *et al*., 1988), or flee from predators. In this context, it has been demonstrated that rats detect and avoid some cat-derived non-volatile chemicals that activate portions of the medial amygdala (reviewed by Dielenberg & McGregor, 2001) and are dependent on the VNO (Panksepp, 1998). The adaptive value of associating intrinsically attractive (possibly rewarding) or aversive VNO-dependent chemicals (or tastes) with odorants (airborne volatiles) in the terrestrial environment might have lead the evolution of the amygdaloid complex of amniotes (Martinez-Garcia *et al*., 2002), and gives functional cohesion to its apparently heterogeneous components (Swanson & Petrovich, 1998; Aggleton & Saunders, 2000).

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Abbreviations

Acb, nucleus accumbens; ACo, anterior cortical amygdala; AOB, accessory olfactory bulb; BLA, anterior portion of the basolateral amygdaloid nucleus; BLP, posterior portion of the basolateral amygdaloid nucleus; BMA, anterior portion of the basomedial nucleus; BSTM, medial bed nucleus of the stria terminalis; Fos-IR, Fos-immunoreactive; MeA, anterior division of the medial amygdala; MePD, posterodorsal division of the medial amygdala; MePV, posteroventral division of the medial amygdala; MOB, main olfactory bulb; NGS, normal goat serum; PLCo, posterolateral cortical amygdala; PMCo, posteromedial cortical amygdala; TBS, Tris-buffered saline; VNO, vomeronasal organ; VO, ventral orbital cortex; VTA, ventral tegmental area.

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