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Differential activation of glomeruli in the ferret's main olfactory bulb by anal scent gland odours from males and females: an early step in mate identification

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Abstract

Peripheral anosmia was previously found to disrupt sex discrimination and partner preference in male and female ferrets. Here we show directly that volatile anal scent gland odourants from male and female ferrets activated overlapping but distinguishable clusters of glomeruli located in the ventral–caudal portion of the main olfactory bulb (MOB) of breeding ferrets of both sexes. No glomerular activation was seen in the accessory olfactory bulb (AOB). The profile of MOB glomerular activation induced in oestrous females by male anal scents was very similar to that induced by direct contact with a male during mating, and oestrogen treatment failed to alter the profile of glomerular activation induced in ovo-hysterectomized females by male anal scents. In rodents, 'atypical' MOB glomeruli, which have dense acetylcholinesterase (AChE) activity in the neuropil, may be activated by body odours from conspecifics. No such AChE-staining 'atypical' glomeruli were found in the ferret's MOB, suggesting that in this carnivore they do not constitute a subset of MOB glomeruli that respond to body odourants. In ferrets of both sexes, volatile body odourants that are detected by the main as opposed to the vomeronasal–AOB accessory olfactory system may play a critical role in mate identification.

Keywords

accessory olfactory bulb; atypical glomeruli; c-fos; pheromones; sex dimorphism; sexual behaviour

Abbreviations

AChE, acetylcholinesterase; AOB, accessory olfactory bulb; D, dorsal; EB, oestradiol benzoate; FB, forebrain; Gl, glomerular layer; IPL, internal plexiform layer; IR, immunoreactivity; M, medial; MHC, major histocompatability complex; MOB, main olfactory bulb; OSN, olfactory sensory neurons; Ovex, ovo-hysterectomized; VNO, vomeronasal organ

Introduction

Body odours signal sex and reproductive status, thereby contributing to mate recognition in many species (Johnston, 1983; Brown & Macdonald, 1985). Some non-volatile body odours are detected by sensory neurons in the vomeronasal organ (VNO) that project to the accessory olfactory bulb (AOB) (Keverne, 1999). In male rodents, lesions of the VNO impaired mating, male–male aggression and hormonal responses to females, all traits that require discrimination of males from females (Halpern & Martinez-Marcos, 2003). Male mice with a null mutation of the *TRP2* gene, which encodes an ion channel required for VNO signal transduction,

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displayed ultrasonic vocalizations and mounting behaviour indiscriminately towards males and females (Leypold *et al*., 2002; Stowers *et al*., 2002), raising the possibility that the VNO–AOB system may be required for sex discrimination in mice.

Body odours are also detected by olfactory sensory neurons (OSN) in the main olfactory epithelium. All OSN expressing a particular odour receptor gene project to one or two glomeruli in the ipsilateral main olfactory bulb (MOB) (Mombaerts *et al*., 1996). Hence, the spatial pattern of MOB glomeruli activated by a particular odourant encodes odour quality and concentration (Xu *et al*., 2000). Mouse urine contains several volatile odours that are detected by OSN and subsequently activate glomeruli and mitral cells in the MOB (Schaefer *et al*., 2001b; Lin & Katz, 2003; Xu *et al*., 2003). Female mice showed different patterns of MOB glomerular activation in response to urinary odours from individual males with different major histocompatability complex (MHC) haplotypes (Schaefer *et al*., 2001b). To our knowledge, there has been no previous investigation of whether body odours from the two sexes activate different groups of MOB glomeruli in any species.

Kelliher & Baum (2001) found that bilateral naris occlusion disrupted the normal preference of ferrets of both sexes to approach opposite-sex conspecifics in Y-maze tests, even when visual plus auditory cues were available to compensate for subjects' olfactory deficit. Here, we investigated whether volatile anal scent gland odourants from male and female ferrets elicit different patterns of MOB or AOB glomerular activation, indexed by the presence of Fos immunoreactivity (IR) in juxtaglomerular cells, in oestrous females (Expt 1) and breeding males (Expt 2). Next, we studied whether stimuli associated with actual mating and exposure to male anal scents produce similar patterns of MOB glomerular activation in females (Expt 3). We then investigated whether oestradiol benzoate (EB), which augmented gonadectomized male and female ferrets' ability to detect low concentrations of anal scents (Woodley & Baum, 2003), modifies MOB glomerular activation induced by male anal scents (Expt 4). Clusters of 'atypical' MOB glomeruli, which have dense acetylcholinesterase (AChE) activity in the neuropil (Zheng *et al*., 1987), appear to overlap partially with the 'modified glomerular complex' that is activated in neonatal rats by maternal pheromones encountered during suckling (Greer *et al*., 1982). In a final study (Expt 5) we invesigated whether AChE-expressing glomeruli exist in the ferret MOB at those locations activated by exposure to anal scents.

Materials and methods

All methods were approved by the Boston University Institutional Animal Care and Use Committee.

Subjects

Adult ferrets (*Mustela putorius furo*) in breeding condition were purchased from Marshall Farms, North Rose, NY, USA, and individually housed in modified rabbit cages under a long-day (16:8 h light–dark) photoperiod. For most experiments, animals were gonadally intact and sexually naïve when exposed to anal scent odours or to odours associated with mating. Animals had not been explicitly exposed to anal scent previously, although they may have encountered such odours at the breeding farm or after they arrived in our Animal Care Facility. The exception was Expt 4, in which females had been ovo-hysterectomized, treated with EB, mated and exposed to male and female anal scents in a previous behavioural experiment (Woodley & Baum, 2003).

Exposure to odours

Oestrous females (n = 13) and breeding males (n = 12) (Expts 1 and 2, respectively) were exposed to female anal scents, male anal scents or unscented air as described below. Anal sacs

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containing scent gland secretions from breeding males and oestrous females were purchased from Marshall Farms and kept frozen at −20 °C until processing. Anal sacs were briefly thawed, and the scent gland secretions were removed, sonicated and refrozen in 50-μL aliquots. On the day of use, aliquots of anal scent from different same-sex animals (two females and three males) were thawed and mixed together in 10 mL of oil vehicle to a final dilution of 1:200. Anal scents from these same donor females and males served as odour stimuli for all experiments. Control subjects were exposed to unscented air blown over 10 mL of oil vehicle. In most instances this vehicle was mineral oil. The exception was Expt 1, in which male anal scent gland secretions were dissolved in sesame oil. In this study unscented air blown over mineral vs. sesame oil yielded low, statistically indistinguishable levels of MOB glomerular activation in oestrous female control subjects ($n = 3$ per diluent). Therefore, these six females were combined into a single control group in which MOB glomerular activation was compared statistically with that of females exposed to either male or female anal scents.

Subjects were food-deprived for 24 h prior to each odour presentation session to minimize urination and defaecation during testing. If either event occurred, the test was discontinued, and subjects were tested on another day. Subjects were placed in a 26 × 46 × 20-cm testing chamber with a Plexiglas lid for 45 min to allow habituation to ambient odours. Next, air was forced continuously at a flow rate of 1375 mL/min for 30 min into a 250-mL flask containing 10 mL of an anal scent gland solution or oil vehicle control (surface area = 5027 mm^2) and then on to the testing chamber through an odour port. The odourant flask was agitated at 1500 r.p.m. Sixty minutes after the end of the stimulus exposure, animals were killed, and olfactory bulbs were processed as described below.

In Expt 3, we examined the pattern of MOB glomerular activation in a group of oestrous females exposed to body odours associated with receipt of mating stimulation from a male. Mating consists of a brief period of anogenital investigation followed by neck gripping and mounting of the female by the male ferret. After a period of pelvic thrusting, penile intromission is achieved, which may last continuously for 1–2 h. Females ($n = 3$) were placed in a clean cage with a male in breeding condition. Ninety minutes after the onset of an intromission (which began an average of 31 min after animals were together), female subjects were killed, perfused and the olfactory bulbs were processed as described below. Control females ($n = 3$) were placed alone in a clean cage and killed 90 min later.

In Expt 4, ovo-hysterectomized females were maintained without sex steroid injections for 1 month and then treated daily with either EB (12 μg/kg s.c., daily) ($n = 3$) or sesame oil vehicle (n=3) for 2–3 weeks and exposed to male anal scent as described above.

Processing of olfactory bulbs

Ninety minutes after the onset of exposure to anal scent, unscented air or penile intromission, animals were killed with an overdose (100 mg/kg) of sodium pentobarbital and perfused with 50 mL of 0.1 M PBS, pH 7.4, followed by 1 L of 4% paraformaldehyde in 0.2 M PB. Previous studies (Wersinger & Baum, 1996; Kelliher *et al*., 1998) indicated that neural Fos-IR in granule cells of the ferret's MOB and in several hypothalamic regions was maximal 90 min following the onset of an olfactory or mating stimulus. Olfactory bulbs were removed and post-fixed in 4% paraformaldehyde for 2 h and then cryoprotected in 30% sucrose until they sank. The right olfactory bulb was sectioned coronally at 30 μm using a freezing sledge microtome. The first eight sections of the rostral portion of the bulb were discarded, and the rest were saved. Every fourth section (120-μm intervals) was processed for Fos-IR as described elsewhere (Kelliher *et al*., 1998) and mounted on slides in exact sequential order. The average diameter of an MOB glomerulus in the ferret is 130 μm, and thus we may have missed scoring the smallest glomeruli and oversampled the largest glomeruli. Our sampling scheme was analogous to that used in mice (Schaefer *et al*., 2001b), in which MOB glomeruli have an average diameter of 85 μm,

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and coronal sections were collected at 72-μm intervals. There is some natural variability in the locations of MOB glomeruli (Schaefer *et al*., 2001a), so maps from several ferrets exposed to the same stimuli within each experiment were averaged.

To estimate the size of each animal's olfactory bulb, the rostro-caudal distance encompassed by the sections scored (see below) for glomerular analysis was estimated. The mean ± SEM distance was 4800 ± 360 μm for oestrous females, and 5700 ± 360 μm for breeding males ($P < 0.05$). In light of this significant difference in olfactory bulb dimensions, statistical comparisons of odourant-induced MOB glomerular activation were not made between the sexes.

Identifying and mapping activated glomeruli

The presence of Fos-IR in juxtaglomerular cells in the ferret MOB was used as an index of glomerular activation, as described previously for mice (Schaefer *et al*., 2001b, 2002). This method, like the uptake of 2-deoxyglucose (Johnson *et al*., 1998), is ideal for mapping the activation of glomeruli induced by body odours because the entire MOB can be examined. By contrast, other techniques, including optical imaging of intrinsic signals (Rubin & Katz, 1999) or imaging of calcium-sensitive dyes (Wachowiak & Cohen, 2001), can only monitor the activation of those glomeruli located in the dorsal MOB. We acknowledge that shortcomings of the present method include the inability to monitor glomerular activation in real time, including our inability to monitor glomerular responses to the presentation of different odourants in the same subject. In the present experiments using ferrets, as in previous studies with mice (Schaefer *et al*., 2001b, 2002), a glomerulus was scored as activated if following exposure to an olfactory stimulus it possessed an arc of Fos-IR juxtaglomerular cells extending 180° in any orientation or two arcs of at least 90° spanning any region but not abutting the external plexiform layer (Fig. 1A).

The location of activated glomeruli was mapped as a function of rostro-caudal distance through the olfactory bulb and radial angle around the MOB (Fig. 1B) using ImageJ v.1.23, a Java image processing program with a plug-in for glomerular analysis (Schaefer *et al*., 2001a,b). Slides were coded so that the person scoring activated glomeruli had no knowledge of any individual subject's identity or treatment. Each section was digitally captured by a Nikon digital camera using Nikon ACT-1 vs. 2.10 software. Activated glomeruli were identified by examining the live image at a magnification of 11.25×, whereupon each activated glomerulus was circled on the captured image. The captured image was processed by the program ImageJ to obtain coordinates of activated glomeruli. Scoring of activated glomeruli began with the first rostral section that included a complete mitral/tufted cell layer and ended with the most caudal section in which the glomerular layer was present. The rostro-caudal position of the first section analysed was offset by 480 μm posterior to the first section that was cut. The radial angle of a section was determined by drawing a vertical axis parallel to the ventral portion of the subependymal layer. Rostrally, the point of origin was one-third of the distance from the dorsal to the ventral mitral/tufted cell layer (Fig. 1A). More caudally, the point of origin was one-third of the distance from the dorsal portion of the lateral olfactory tract to the ventral mitral/tufted cell layer.

Making maps of glomerular activation

The data on activated glomeruli for each subject were binned and kernelled with ToMatrix v. 1 software (downloaded from the Restrepo Laboratory homepage at http://www.uchsc.edu/ctrsinst/rmtsc/restrepo), adapted from use in the mouse MOB (Schaefer *et al*., 2001b) for use in the ferret MOB by changing the distance between sections from 72 to 120 μm. Activated glomeruli were arrayed into bins of 10° radial angle and 120 μm rostral–caudal distance. To decrease error attributable to mapping as well as variability in glomerular localization (Schaefer

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et al., 2001a), for each subject the value for each bin was replaced with the sum of the number of activated glomeruli in a 3 × 3-bin kernel centred on that bin. Group averages were computed, and these kernelled data were then visualized as a colour-contour plot constructed using SigmaPlot 2001 software.

Statistical analysis

As described above, the data for each subject were summarized as a rectangular array of bins, each containing the number of activated glomeruli within a volume of the MOB glomerular zone defined by 10° radial angle and 120 μm rostral–caudal distance These results were analysed using non-parametric, two-tailed, bin-wise Mann–Whitney *U*-tests provided by the ToMatrix v.1 software (Schaefer *et al*., 2002) to determine whether between-group differences in spatial patterns of glomerular activation were statistically significant. We asked whether the number of activated glomeruli in 3×3-bin kernels centred on each bin differed between two treatment groups by sorting the values and computing *U* in nine adjacent bins. Probability values were then adjusted for the large number of bin-wise Mann–Whitney *U* comparisons made by using Bonferroni corrections in order to reduce the incidence of Type I statistical errors. This conservative statistical procedure would have minimized the number of statistically significant group differences obtained. These statistical results were portrayed using colour-contour plots similar to those used to visualize areas of glomerular activation.

Identifying ‘atypical’ glomeruli (Expt 5)

Breeding adult (five male, four female) as well as 15-day-old (six male, six female) ferrets were perfused (see details above). The brains were removed and cryoprotected, whereupon the right olfactory bulbs were sectioned coronally at 30 μm, and every section was processed for AChE histochemistry (Hedreen *et al*., 1985). Sections through the olfactory bulbs of five adult male mice, in which ‘atypical’ MOB glomeruli abutting the AOB appear as dark patches of densely AChE-staining neuropil (Weruaga *et al*., 2001), were included as a positive control.

Results

Male and female anal scents activated distinguishable groups of glomeruli in the ventral MOB

In both female (Expt 1) and male (Expt 2) subjects, exposure to male as opposed to female anal scents maximally activated partially overlapping but distinguishable clusters of MOB glomeruli (Figs 2B and C, and 3B and C), whereas in ferrets exposed to unscented air relatively few glomeruli were activated (Figs 2A and 3A). Statistical comparisons showed that exposure to female anal scent significantly activated a relatively small cluster of glomeruli in the ventral aspect of the caudal MOB in both sexes relative to unscented air controls (Figs 2E and 3E). Exposure to male anal scent significantly activated a wide band of glomeruli in the ventral MOB of both sexes relative to controls (Figs 2F and 3F). In subjects of both sexes, female anal scent activated a relatively small cluster of glomeruli that was also activated by male anal scent whereas male anal scent activated a more extensive ventral area than female anal scent (Figs 2D and 3D). Exposure to male anal scent also activated areas outside the ventral zone. This was particularly evident in female subjects exposed to male anal scent in which a number of rostral and dorsal areas also showed significant activation compared with controls (Fig. 2F). Exposure to anal scent gland odourants never induced Fos-IR in the glomerular layer of the AOB (data not shown).

Mating activated glomeruli in the ventral MOB of oestrous females

The receipt of mating stimulation (Expt 3) maximally activated a cluster of glomeruli in the ventral MOB of oestrous females (Fig. 4B). The location and size of this cluster of maximally activated glomeruli was very similar to that induced in other female subjects solely by exposure

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to male anal scent (Fig. 2C). Body odours, including male anal scent, that are detected during the receipt of a neck grip, mount and penile intromission probably represent the maximal olfactory stimulus that an oestrous female can receive from a male. These observed similarities between mating- and odour-induced glomerular activation in the ventral MOB suggest that exposure to a 1:200 dilution of male anal scent (Expts 1 and 2) activated the maximal number of OSN capable of responding to these odourants. It is interesting to note that mating, but not exposure to male anal scents alone, also maximally activated clusters of glomeruli located in the dorsal and rostral MOB of oestrous female subjects (compare Figs 4B and 2C; see also Fig. 5A and B). This additional effect of mating may have resulted from the actions of male body odourants in addition to anal scents and/or in response to the activation of centrifugal inputs to the MOB associated with the receipt of neck gripping, mounting and penile intromission.

No effect of EB treatment on male anal scent-induced glomerular activation in ovo-hysterectomized females

In ovo-hysterectomized females (Expt 4), male anal scent maximally activated a widespread ventral caudal area of the glomerular layer (Fig. 5A and B) that was very similar to that seen in gonadally intact oestrous females (Fig. 2C). There were no significant statistical differences in the patterns of glomerular activation elicited by male anal scent between ovariectomized females injected with sesame oil vehicle or EB (Fig. 5C).

Ferrets lack ‘atypical’ glomeruli in the MOB

No dense AChE staining of ‘atypical’ glomeruli was ever observed in coronal sections taken throughout the MOB of either adult or P15 ferrets of either sex (Fig. 6A), whereas such ‘atypical’ MOB glomeruli were identified in MOB sites abutting the AOB of all five mice examined (Fig. 6B). Although ferrets lacked AChE staining that is characteristic of ‘atypical’ glomeruli, they had a laminar distribution of AChE staining in the glomerular and internal plexiform layers of the MOB similar to that seen in mice (Fig. 6A and B).

Discussion

Our results suggest that the main olfactory system is able to distinguish volatile anal scent gland odourants derived from male and female ferrets. The subjects studied in Expts 1 and 2 had not had previous mating experience, suggesting that the observed differences in the patterns of MOB glomerular activation shown in response to male vs. female anal scents probably did not depend on previous association of these odourants with mating stimulation. In previous studies, ferrets preferred to approach volatile body odours (Kelliher & Baum, 2002) as well as anal scent gland odourants (Clapperton *et al*., 1988) from opposite-sex individuals, suggesting that anal scents may contribute to sex discrimination and heterosexual partner preference. A further test of the role of the main olfactory system in ferret mate identification involved occlusion of the nares, which blocked the access of odours to OSN (Kelliher & Baum, 2001). This procedure eliminated the preference of both male and female ferrets to approach opposite-sex conspecifics in Y-maze tests even when subjects had physical contact with conspecifics after each operant trial. In mice, such direct contact was required for activation of AOB mitral cells (Luo *et al*., 2003). The observed lack of activation of AOB glomeruli after exposure to anal scents (Expts 1 and 2) or to additional body odours associated with mating (Expt 3) is consistent with previous studies (Wersinger & Baum, 1997; Kelliher *et al*., 1998) on ferrets of both sexes showing that neither exposure to odours from soiled bedding nor mating itself augmented the number of Fos-IR granule cells in the AOB. These results are also consistent with a recent study (S. K. Woodley, A. L. Cloe, P. Waters and M. J. Baum, unpublished results) in which surgical removal of the vomeronasal organ failed to disrupt the preference of oestrogen-primed female ferrets to approach volatile body odours from anaesthetized males in Y-maze tests. Taken together, our results support the view that the accessory olfactory system

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does not contribute to mate identification in ferrets. Instead, the differential activation of clusters of MOB glomeruli by male vs. female anal scent gland odourants, perhaps acting in consort with other body odours, may represent an initial step in sex discrimination leading to the identification of potential mates.

The ventral-caudal MOB had the greatest density of maximally activated glomeruli in ferrets of both sexes that were exposed to anal scent gland odourants and in females after mating. A similar region of the MOB was maximally activated in female mice after exposure to male urinary odours as measured by *c-fos* mRNA in juxtaglomerular cells (Schaefer *et al*., 2001b, 2002) and by fMRI (Xu *et al*., 2003). It was suggested (Schaefer *et al*., 2001b, 2002) that different patterns of MOB glomerular activation contribute to recognition of males with different MHC haplotypes; however, such MOB glomerular responses to urinary odours may also contribute to sex discrimination in mice. Support for this view is provided by the observation (Lin & Katz, 2003) that some MOB mitral cells of female mice selectively responded to urinary odours from males vs. females. We investigated whether ‘atypical’ glomeruli, which have been hypothesized selectively to process socially relevant odours in rodents (Zheng *et al*., 1987; Lin *et al*., 2004), might be present in those regions of the ferret’s MOB that were differentially activated by anal scents derived from males and females. The apparent absence of ‘atypical’ MOB glomeruli in ferrets, assessed using AChE staining, should be confirmed in future studies using additional markers, including placental antigen (Shinoda *et al*., 1989) and cGMP-stimulated phosphodiesterase 2 (Juilfs *et al*., 1997). However, the data in hand suggest that ‘atypical’ glomeruli such as those seen in rodent species do not exist in ferrets and thus do not constitute a subset of MOB glomeruli that respond to body odourants.

The present results in the ferret together with data for the mouse (Schaefer *et al*., 2001b, 2002) suggest that glomeruli in the ventral MOB may be specialized to process body odours. Most mitral/tufted cells of the MOB project to cortical areas mediating olfactory perception; however, a subset of these projection neurons also innervate the anterior–cortical amygdala (Scalia & Winans, 1975). The anterior–cortical amygdala projects to the medial amygdala (Kevetter & Winans, 1981; Coolen & Wood, 1998), which in turn projects to areas that regulate reproductive behaviours and physiology, including the preoptic area and ventromedial hypothalamus. Hence, it is possible that mitral/tufted cells abutting ventral MOB glomeruli may selectively project to the anterior–cortical amygdala. Studies are needed to determine whether the anterior–cortical amygdala receives selective inputs from ventral MOB glomeruli, which in turn are innervated by OSN that detect body odours.

Volatile anal scent gland secretions of ferrets consist of several compounds, of which seven have been identified (Clapperton *et al*., 1988). Both males and females produce all seven compounds, with females having significantly more sulphur-containing compounds (2,3-dimethyl-thietane, and 3,4-dimethyl-1,2-dithiolane) and males having more nitrogen-containing indole. Female anal scents maximally activated a single, discrete cluster of ventral MOB glomeruli in ferrets of both sexes. Male anal scents maximally activated the same cluster plus additional glomeruli distributed across the ventral MOB, in addition to clusters of glomeruli located in the rostral–dorsal MOB. The differences in glomerular activation induced by male and female anal scent could reflect differences in the initial OSN input to the glomerular layer, as well as differences in the processing of glomerular activity. In this study, we used the presence of Fos protein in juxtaglomerular interneurons as an index of glomerular activation. This index reflects cumulative neuronal activity over several minutes, raising the possibility that the observed Fos signal represents more than the initial OSN input to MOB glomeruli. Juxtaglomerular cells include periglomerular, short axon and external tufted interneurons (Shipley & Ennis, 1996). Juxtaglomerular cells contribute to intraglomerular modulation via inhibitory dendro-axonic synapses with OSN (Aroniadou-Anderjaska *et al*., 2000; Ennis *et al*., 2001), and reciprocal dendro-dendritic synapses with mitral/tufted cells

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(Shipley & Ennis, 1996). Juxtaglomerular cells also are modulated by interactions with juxtaglomerular cells in other glomeruli. Short axon cells project to glomeruli as far as 20–30 glomeruli away, where they activate inhibitory periglomerular cells and excite other short axon cells and external tufted cells (Aungst *et al*., 2003). Hence, a wide network of interglomerular modulation exists. The ability of male and female anal scents to activate distinguishable clusters of MOB glomeruli corresponds to the ability of male and female ferrets to discriminate between them in behavioural tests in which subjects' spontaneous interest in these odours motivated their performance (Woodley & Baum, 2003). A similar correlation between distinguishable patterns of MOB glomerular activation and the ability of rats spontaneously to discriminate odours in behavioural tests was obtained with two enantiomers of carvone that have minimal structural differences (Linster *et al*., 2001).

Oestradiol, secreted by the ovaries in females or derived from neural aromatization of testosterone in males, augmented the ability of gonadectomized ferrets of both sexes to detect low concentrations of anal scents (Woodley & Baum, 2003). In light of the correlation between distinguishable patterns of MOB glomerular activation and subjects' ability to discriminate behaviourally between the two odours (Linster *et al*., 2001), we investigated whether administering oestradiol would alter the pattern of glomerular activation seen in ovo-hysterectomized females just as it enhanced their ability to detect male anal scents. No such effect was seen, which raises the question of whether EB treatment may have increased ferrets' behavioural responsiveness to low concentrations of anal scents by acting downstream from MOB glomeruli at other MOB or forebrain areas. A precedent for such an action of a sex steroid was previously observed (Kelliher *et al*., 1998): testosterone treatment significantly augmented MOB granule cell as well as bed nucleus of the stria terminalis Fos-IR responses induced in gonadectomized ferrets by odours from soiled oestrous female bedding.

The VNO–AOB accessory olfactory system has recently received much attention as a prime determinant of sex discrimination in male mice (Leypold *et al*., 2002; Stowers *et al*., 2002). Although not ruling out some role for the accessory olfactory system, our demonstration that male vs. female anal scent gland odourants induced distinguishable patterns of MOB glomerular activity in ferrets points to a possible role of the main olfactory system in heterosexual mate identification in this carnivore. Reports that female mice show distinguishable patterns of MOB glomerular activation (Schaefer *et al*., 2001b, 2002) and discriminate behaviourally (Yamazaki *et al*., 1983) between urinary odours from males with different MHC loci raise the possibility that the main olfactory system also contributes to mate recognition in rodents. This view is further supported by the report (Ma *et al*., 2002) that selective destruction of OSN, while sparing the VNO, eliminated the ability of female mice to identify urinary odours from males.

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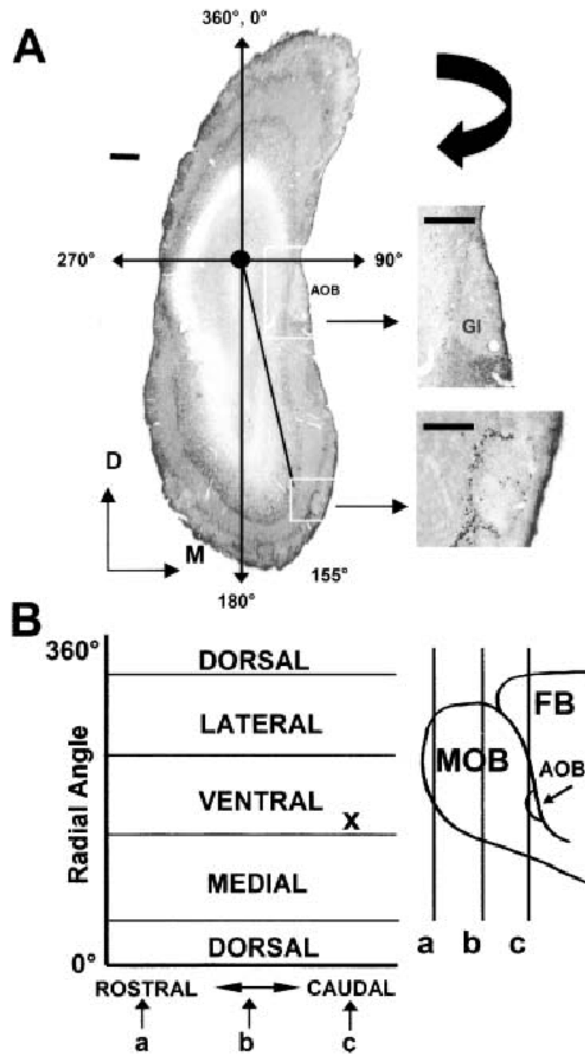

Fig. 1.

Identifying and mapping activated MOB glomeruli based on the presence of Fos-IR juxtaglomerular cells following exposure to anal scents. (A) A representative coronal section (taken at level 'c' from the diagram in B) showing Fos-IR in juxtaglomerular cells in the MOB of an oestrous female ferret after exposure to volatile components of male anal scent. The upper inset shows the AOB at a higher magnification to illustrate the absence of Fos-IR cells in the glomerular layer. The lower inset shows the MOB at a higher magnification to illustrate the presence of an activated glomerulus with a ≥ 180° arc of Fos-IR juxtaglomerular cells. The upper boundary of a second, non-activated glomerulus in which the arc of Fos-IR juxtaglomerular cells did not exceed 180° is also shown at the bottom of the inset. The radial angle corresponding to each activated glomerulus was determined according to a vertical axis and point of origin, as shown. (B) The rostro-caudal location and radial angle of each activated glomerulus was used to map patterns of odour-induced glomerular activity. The location of the activated glomerulus shown in A is shown with an X on the map. Letters a–c indicate the rostro-caudal location of different coronal sections in the sagittal diagram of the olfactory bulb and the map. D, dorsal; M, medial; FB, forebrain; Gl, AOB glomerular layer. Scale bars, 100 µm.

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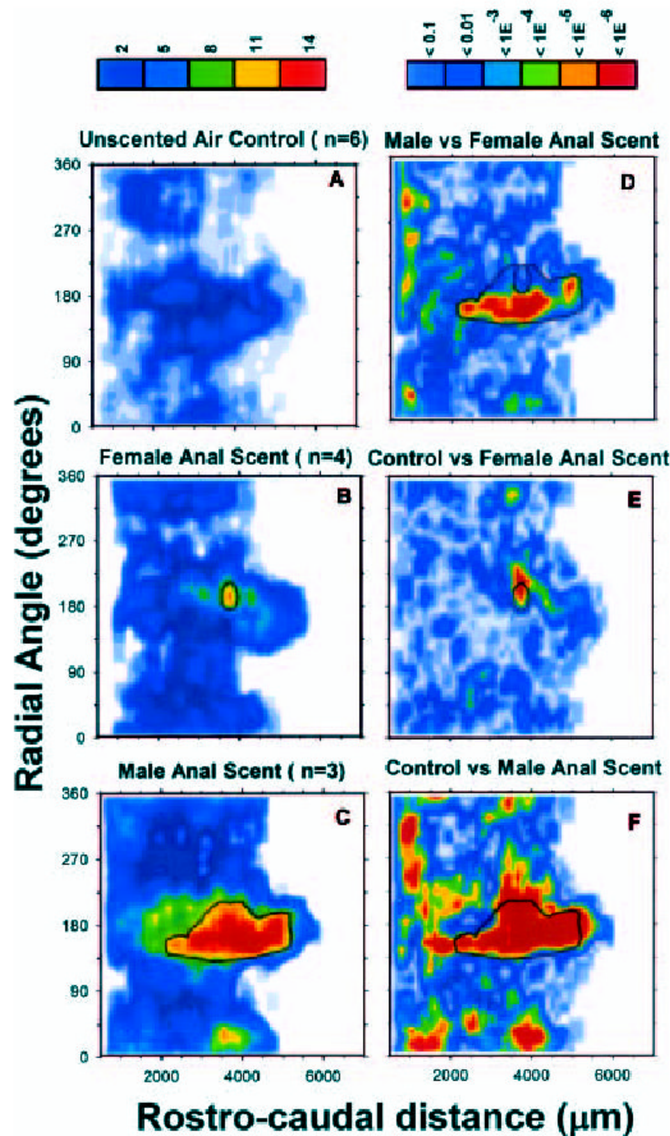

Fig. 2.

Patterns of glomerular activation in the MOB of gonadally intact, oestrous female ferrets after exposure to male vs. female anal scents. Data are plotted in bins defined by radial angle and rostro-caudal distance through the olfactory bulb. (A–C) The average number of activated glomeruli per bin after exposure to unscented air (A), female anal scent (B) or male anal scent (C); scale above the left column indicates the number of activated glomeruli per bin. No activated glomeruli were seen in white areas. (D–F) *P*-values from bin-wise Mann–Whitney *U*-tests comparing differences between female vs. male anal scent (D), control vs. female anal scent (E) and control vs. male anal scent (F). The scale above the right column indicates *P*-values; $P \leq 1 \times 10^{-5}$ was significantly different after Bonferroni correction. The black outlines in B and C indicate the areas of maximal activation in response to female and male anal scent, respectively. These black outlines are superimposed on the Mann–Whitney *U*-plots (D–F) and indicate whether areas of maximal activation were significantly different between groups.

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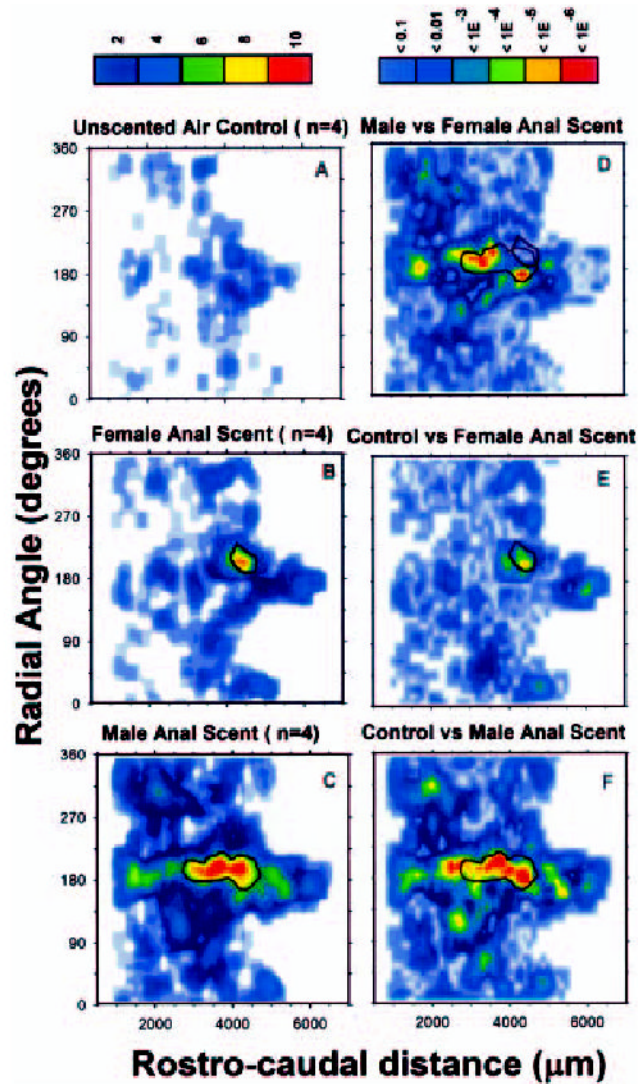

Fig. 3.

Patterns of glomerular activation in the MOB of gonadally intact, breeding male ferrets after exposure to male vs. female anal scents. Data are plotted in bins defined by radial angle and rostro-caudal distance through the olfactory bulb. (A–C) The average number of activated glomeruli per bin after exposure to unscented air (A), female anal scent (B) or male anal scent (C); scale above the left column indicates the number of activated glomeruli per bin. No activated glomeruli were seen in white areas. (D–F) *P*-values from bin-wise Mann–Whitney *U*-tests comparing differences between female vs. male anal scent (D), control vs. female anal scent (E) and control vs. male anal scent (F). The scale above the right column indicates *P*-values; $P \leq 1 \times 10^{-5}$ was significantly different after Bonferroni correction. The black outlines in B and C indicate the areas of maximal activation in response to female or male anal scent, respectively. These black outlines are superimposed on the Mann–Whitney *U*-plots (D–F) and indicate whether areas of maximal activation were significantly different between groups.

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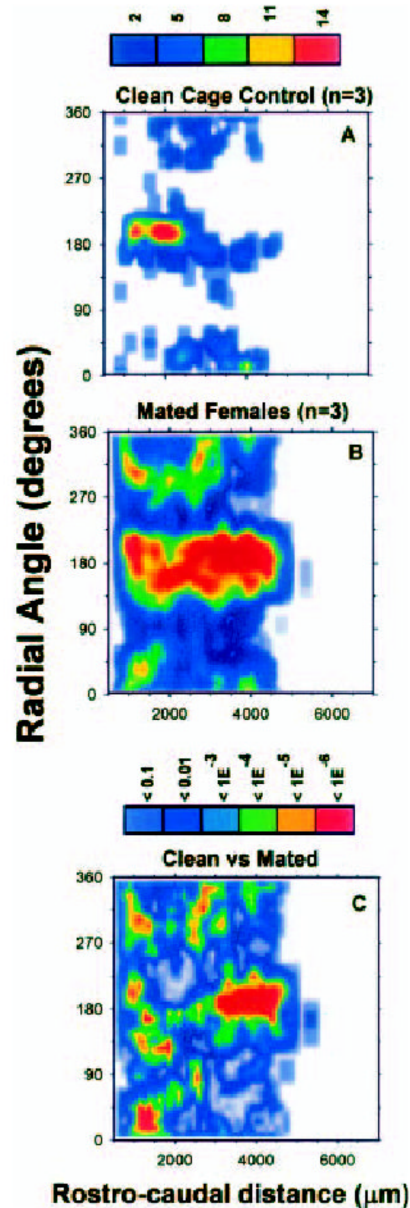

Fig. 4.

Patterns of glomerular activation in the MOB of gonadally intact, oestrous female ferrets after mating. Data are plotted in bins defined by radial angle and rostro–caudal distance through the olfactory bulb. (A and B) The average number of activated glomeruli per bin after exposure to a clean cage (A) or after receiving a neck grip, mount and intromission from a male (B). The scale above A indicates the number of activated glomeruli per bin. No activated glomeruli were seen in white areas. (C) *P*-values from bin-wise Mann–Whitney *U*-tests comparing differences between the two conditions. The scale above C indicates *P*-values; $P \leq 1 \times 10^{-5}$ was significantly different after Bonferroni correction.

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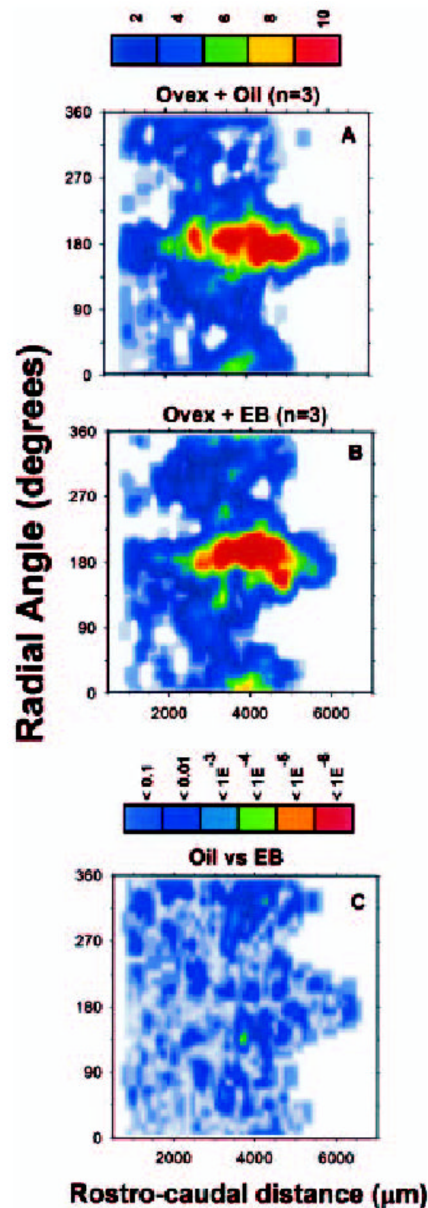

Fig. 5.

Effect of oestrogen treatment on patterns of MOB glomerular activation induced by exposure to male anal scents in ovo-hysterectomized (Ovex) female ferrets. Data are plotted in bins defined by radial angle and rostro-caudal distance through the olfactory bulb. (A and B) The average number of activated glomeruli per bin after exposure to male anal scents in ovo-hysterectomized female ferrets previously injected daily with either oil vehicle (A) or oestradiol benzoate (EB) (B). The scale above A indicates the number of activated glomeruli per bin. No activated glomeruli were seen in white areas. (C) *P*-values from bin-wise Mann–Whitney *U*-tests comparing differences between the two conditions. The scale above C indicates *P*-values; $P \leq 1 \times 10^{-5}$ was significantly different after Bonferroni correction.

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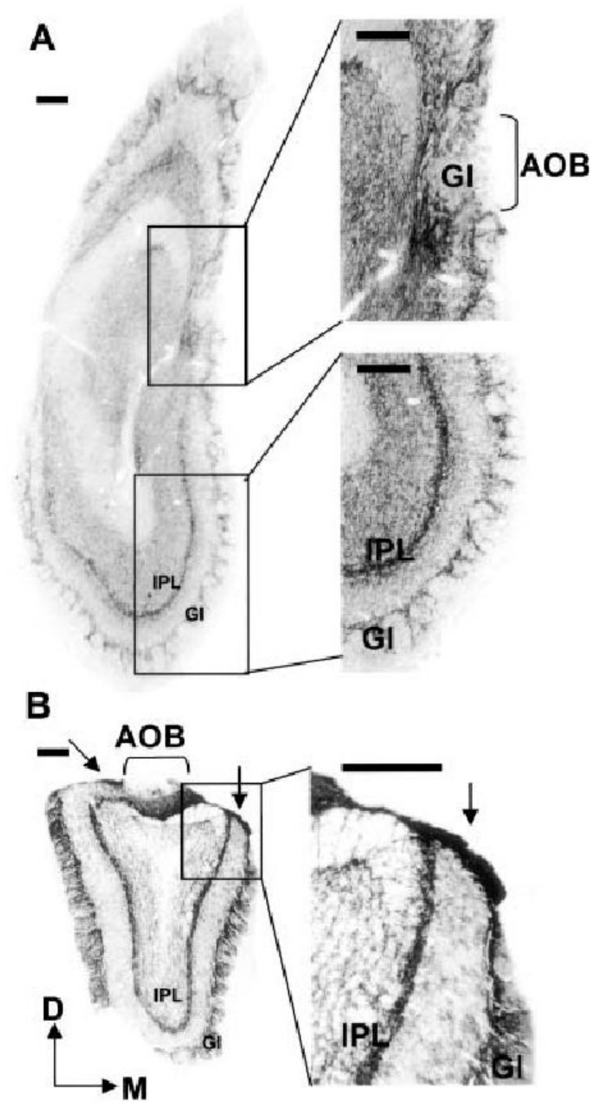

Fig. 6.

Absence of ‘atypical’ glomeruli in the MOB of ferrets. Representative coronal sections show AChE histochemistry in the MOB and AOB of an oestrous female ferret (A) and a male mouse (B). Insets show boxed areas at higher magnification. Note the presence of AChE staining in the glomerular (Gl) and internal plexiform layers (IPL) in both ferret and mouse. Arrows in B indicate the location of densely staining ‘atypical’ glomeruli in the mouse, located adjacent to the AOB. D, dorsal; M, medial. Scale bars, 250 μm.