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Evidence for a role of early oestrogens in the central processing of sexually relevant olfactory cues in female mice

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Abstract

We previously found that female aromatase knockout (ArKO) mice showed less investigation of socially relevant odours as well as reduced sexual behaviour. We now ask whether these behavioural deficits might be due to an inadequate processing of odours in female ArKO mice. Therefore, we exposed female ArKO mice to same- and opposite-sex urinary odours and determined the expression of the immediate early gene *c-Fos* along the main and accessory olfactory projection pathways. We included ArKO males in the present study as we previously observed that they show female-typical detection thresholds of urinary odours, suggesting a role for perinatal oestrogens in these behavioural responses. No sex or genotype differences were observed in the olfactory bulb after urine exposure. By contrast, sex differences in *c-Fos* responses were observed in wild-type (WT) mice following exposure to male urine in the more central regions of the olfactory pathway; only WT females showed a significant Fos induction in the amygdala, central medial pre-optic area and ventromedial hypothalamus. However, ArKO females did not show a *c-Fos* response to male odours in the ventromedial hypothalamus, suggesting that the processing of male odours is affected in ArKO females and thus that oestrogens may be necessary for the development of neural responses to sexually relevant odours in female mice. By contrast, *c-Fos* responses to either male or oestrous female urine were very similar between ArKO and WT males, pointing to a central role of androgen vs. oestrogen signalling in the male circuits that control olfactory investigation and preferences.

Introduction

In many mammalian species, including mice, odours provide essential information about the sex, social and reproductive status of conspecifics (Brown, 1979). They induce hormonal changes, play a key role in mate recognition and may thus be very important for the expression of reproductive behaviours (reviewed in Bakker, 2003). These socially relevant odours may be detected by either the main or the accessory olfactory system. The main olfactory system is generally used to detect volatile odourants derived from food, predators and potential mates, among many sources (Firestein, 2001), whereas the accessory olfactory system detects non-volatile odours that influence reproductive and aggressive behaviours in rodent species, including the mouse (Keverne, 1999).

The accessory olfactory system has sexually dimorphic characteristics (morphological and functional) along its projection pathway, indicating an important role for sex steroid hormones in its development and functioning (Bakker *et al.*, 1996; Bressler & Baum, 1996; Guillamon & Segovia, 1997). For instance, sex differences in immediate early gene (*c-Fos*) responses were observed along the entire accessory olfactory projection pathway when mice were exposed to bedding soiled by gonadally intact males (Halem *et al.*, 1999). These sex differences may reflect the perinatal action of oestrogens in the male nervous system as male rats treated neonatally with an aromatase inhibitor showed female-typical *c-Fos* responses when exposed to male odours (Bakker *et al.*, 1996). By contrast, fewer reports exist on sex differences and thus sex hormone action for the main olfactory system but sex differences have been observed in olfactory sensitivity in pigs (Dorries *et al.*, 1995) and mice (Baum & Keverne, 2002).

We recently observed that female aromatase knockout (ArKO) mice, which carry a targeted mutation in the aromatase gene and as a result cannot convert androgens into oestrogens, showed decreased levels of olfactory investigation of conspecific odours in a Y-maze as well as decreased female sexual behaviour when paired with a sexually active male (Bakker *et al.*, 2002a). In addition, ArKO females failed to show significant dishabituation responses when exposed to a dilution series of urine (Pierman *et al.*, 2006a). As olfaction is essential for the expression of sexual behaviours in mice (Thompson & Edwards, 1972; Edwards & Burge, 1973; Keller *et al.*, 2006a,b), we hypothesized that the decrease in sexual behaviour may be due to an inability of ArKO female mice to respond to sexually relevant olfactory cues. Therefore, we exposed female ArKO mice to same-sex and opposite-sex urinary odours and determined *c-Fos* responses along the main and accessory olfactory projection pathway. We also included ArKO males in this study as we observed that ArKO males showed female-typical detection thresholds of urinary odourants, suggesting that sex differences in urine detection thresholds reflect the perinatal action of oestrogens in the male nervous system (Pierman *et al.*, 2006a). We expected that the detection of odours would not be affected at the level of the olfactory bulb in ArKO mice as they are

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able to discriminate between male and female urinary odours (Pierman *et al.*, 2006a; Wesson *et al.*, 2006) but that more central *c-Fos* responses would be affected.

Materials and methods

Animals

The ArKO mice were generated by targeted disruption of exons 1 and 2 of the *Cyp 19* gene (Honda *et al.*, 1998). Heterozygous males and females of the C57Bl/6j strain were bred to generate wild-type (WT), heterozygous and homozygous-null (ArKO) offspring. Mice were genotyped by polymerase chain reaction analysis of tail DNA (for more detailed description see Bakker *et al.*, 2002b). All breeding and genotyping were performed at the Centre for Cellular and Molecular Neurobiology, University of Liège, Belgium. Food and water were always available *ad libitum* and the temperature was maintained at 22 °C.

Adult WT and ArKO mice of both sexes were gonadectomized under general anaesthesia through an intraperitoneal injection of a mixture of ketamine (80 mg/kg per mouse) and medetomidine (Domitor, Pfizer, 1 mg/kg per mouse). Mice received atipamezole (Antisedan, Pfizer, 4 mg/kg per mouse, subcutaneously) at the end of surgery in order to antagonize medetomidine-induced effects, thereby accelerating their recovery. Mice were then placed in individual cages and treated daily with oestradiol benzoate (OB) (5 μg in 0.05 mL/mouse) by subcutaneous injection in the neck at least 2 weeks before being exposed to odours. Males and females were housed in two separate rooms under a reversed light/dark cycle (12/12 h).

All experiments were conducted in accordance with the guidelines set forth by the National Institutes of Health *Guiding Principles for the Care and Use of Research Animals* and were approved by the Ethical Committee for Animal Use of the University of Liège.

Urine collection

Urine was collected from 10 C57Bl6/j males, which were left gonadally intact. Oestrous female urine was collected from 10 ovariectomized female mice, which had an oestradiol implant (diluted 1 : 1 with cholesterol; for more details see Bakker *et al.*, 2002a) and were injected with 500 μg progesterone at 2–4 h prior to urine sampling. Urine was collected by holding the mouse by the scruff of the neck over a funnel, taking care that no faecal contamination of the urine occurred. Same urine stimulus samples were pooled and subsequently aliquoted in 500-μL Eppendorf vials and stored at −80 °C until use. Urine was pooled because it has been shown to be difficult to obtain sufficient urine from one single donor, particularly in oestrous females, as well as to minimize individual variability between the urine donors.

Urine exposure

After 2 weeks of OB treatment, mice were trained daily, for 1 week, in the manipulation used for urine exposure while continuing to receive OB treatment. During the dark phase of the light/dark cycle, animals were taken out of their home cage and received 30 μL of deionized water onto their nose and were then placed back in their cage. On the day of urine exposure, they were divided into three different groups depending on the odour stimulus that they were going to be exposed to. Group 1 was exposed to intact male urine (six of each sex and each genotype), group 2 to oestrous female urine (six of each sex and genotype) and group 3 to deionized water to serve as control (WT, six females and five males; ArKO, two females and six males).

Ninety minutes later, animals were anaesthetized with the same mixture of ketamine/medetomidine used for gonadectomy and perfused transcardially with saline followed immediately by 4% cold paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 2 h. Brains were then cryoprotected in 30% sucrose/phosphate-buffered saline solution and, when sunken, frozen on dry ice and kept at −80 °C. Sections of 30 μm were cut on a Leica cryostat. Olfactory bulbs and forebrains were cut coronally, saved in antifreeze solution and maintained at −20 °C for later immunohistochemistry.

Immunohistochemistry

Every fourth section of brain and olfactory bulb was processed for Fos immunoreactivity as previously described (Halem *et al.*, 2001). All incubations were carried out at room temperature (21°C) and all washes of tissue sections were performed using Tris-buffered saline or phosphate-buffered saline. Briefly, sections were pre-incubated for 3 h in 7.5% normal goat serum (NGS) in Tris-buffered saline containing 0.1% Triton X-100. Sections were then incubated overnight with a rabbit polyclonal anti-*c-Fos* antibody (1 : 3000 in Tris-buffered saline containing 0.1% Triton X-100/2% NGS; Santa Cruz SC-52) followed by an incubation for 1 h in a goat anti-rabbit biotinylated antibody (1 : 200 in Tris-buffered saline containing 0.1% Triton X-100/2% NGS; Dako Cytomation, Denmark). To eliminate endogenous peroxidase activity, sections were incubated for 30 min in phosphate-buffered saline containing H_2O_2 at a final concentration of 3%. Sections were then incubated for 45 min in avidin–biotin complex (ABC, Vector Laboratory) and reacted for 5 min with 3,3′diaminobenzidine tetrahydrochloride containing nickel chloride (Vector Laboratory). Sections were washed, mounted on gelatin-coated slices, dried, left in SafeSolv for 5 min (Labonord) and coverslipped using SafeMount (Labonord).

Data analysis

Numbers of Fos-immunoreactive (ir) cells were counted in several brain areas implicated in the accessory [accessory olfactory bulb (AOB), anterior medial amygdala (MeA), posterior part of the medial amygdala, medial part of the bed nucleus of the stria terminalis, medial part of the medial pre-optic nucleus, central part of the medial pre-optic nucleus (MPoC), and ventromedial hypothalamic nucleus (VMH)] and main [main olfactory bulb (MOB), piriform cortex and anterior cortical amygdaloid nucleus (ACo)] olfactory pathways. Numbers of Fos-ir cells were quantified by an experimenter, who was blind to the experimental treatment of the mice, using computer-assisted image analysis. Sections were digitized through a video camera attached to the microscope (40× objective for olfactory bulb areas, 20× objective for all other brain areas) and Fos-ir cells were counted with a PC-based image analysis system using the particle-counting protocol of the NIH Image program (Version 1.37; Wayne Rasband, NIH, Bethesda, MD, USA). Digital images were made binary and a manual threshold was used for discriminating the labelled material from the background. With a 20× objective, exclusion thresholds were set at 10 (low threshold) and 100 (high threshold) pixels to remove from the counts dark objects that were not the same size as a cell nucleus (30 and 300, respectively, with a 40× objective for the bulb sections).

Brain structures were identified based on the atlas of the mouse (Paxinos & Franklin, 2001) and Fos-ir cells were measured in one

entire field (computer screen; length, 0.536 mm; height, 0.360 mm; field placed in landscape position in each case) that was placed in a standardized manner based on pre-defined anatomical landmarks in the sections (e.g. edge of ventricles or prominent fibre tracts).

The areas that were quantified are described in the following sections.

MOB and AOB

MOB

Anterior sections of the olfactory bulb were used for the quantification of the MOB (Plate 01 of the mouse atlas; interaural, 8.08 mm; bregma, 4.28 mm). A computer field was placed at the ventral and dorsal parts of the granular layer of the MOB on the dorso-ventral axis. Fos was quantified at both the dorsal and ventral parts of the MOB as previous studies (Schaefer *et al.*, 2001; Martel *et al.*, 2007) have shown that volatile urinary odours preferentially activate the ventral MOB. For each level, the entire computer field with a 40× objective (0.0965 mm^2) served to quantify the numbers of Fos-ir cells.

AOB

The Fos-ir cells were counted in two successive sections where both mitral and granular cell layers of the AOB were present: the most anterior section (S1) and a section located 60 μm more caudally than S1 (S2). In each section, the quantification field for the mitral layer was located just above the dorsal lateral olfactory tract and the granular layer between the dorsal lateral olfactory tract and the intrabulbar part of the anterior commissure. For each level, the entire computer field with a 40× objective was always quantified (0.0965 mm^2). For the mitral and granular cell layers, the numbers of Fos-ir cells in S1 and S2 were averaged and used as dependent measures.

Central brain areas receiving olfactory inputs

Piriform cortex

The section where the anterior commissure reaches its largest extension was used for the quantification of Fos-ir cells in the piriform cortex. The quantification field was placed at the ventral part of the piriform cortex parallel to the floor of the brain. Numbers of Fos-ir cells on both sides of the brain were averaged for the piriform cortex. However, exposure to male or oestrous female urine did not induce a significant Fos response and therefore the results for this brain area will not be presented in the Results.

ACo

The ACo was localized in a section corresponding to Plate 40 of the mouse atlas (interaural, 2.74 mm; bregma, −1.06 mm). The computer field with a 20× objective was first placed on the MeA (defined by the optic tract and the floor of the brain) and then the quantification field was moved one half field laterally. Quantification of the ACo was made on both sides of the brain and numbers of Fos-ir cells were averaged.

MeA

The Fos-ir cells in the MeA were quantified in a section corresponding to Plate 41 of the mouse atlas (interaural, 2.58 mm, bregma, −1.22 mm) at both sides of the brain in a field defined by the optic tract and the floor of the brain. The mean of Fos-ir cells in these two fields with a 20× objective was used as the measure for the MeA.

MePV and MePD

The posteroventral part of the medial amygdala (MePV) and posterodorsal part of the medial amygdala (MePD) are found caudally to the MeA. The quantification field of the MePV with a 20× objective was localized at the floor of the brain nearly adjacent to the optic tract. The field for the MePD was more dorsal, halfway up, and adjacent to the optic tract. Numbers of Fos-ir cells in MePV and MePD were counted in each side of the section and averaged.

Medial part of the bed nucleus of the stria terminalis

Fos expression was quantified on both sides at a rostral level with a 20× objective (Plate 32 of the mouse atlas; interaural, 3.70 mm; bregma, −0.10 mm). The quantification field (0.193 mm^2) was localized under the lateral ventricle, between the anterior commissure, fornix and fibres of the stria terminalis. However, no significant induction of Fos was observed in this brain following exposure to either male or oestrous female urine. Therefore, no data for this brain area will be presented here.

MPoC

Quantification for this brain area occurred in a section corresponding approximately to Plate 31 of the mouse atlas (interaural, 3.82 mm; bregma, 0.02 mm). The quantification field with a 20× objective was first placed in the corner formed by the lateral edge of the third ventricle and the floor of the brain. The field was then moved two fields dorsally along the third ventricle and one field laterally. Quantification was made on both sides of the brain and the average of these two sides gave numbers of Fos-ir cells for the central part of the pre-optic area.

VMH

The VMH was quantified in one section corresponding to Plate 42 of the mouse atlas. The computer field was placed along the third ventricle on the floor of the brain and then moved one half field dorsally and one field laterally. Quantification was made on both sides of the brain with a 20× objective and the average was used as the measure for the VMH.

Statistical analysis

All data were analysed using ANOVA with the number of Fos-ir cells as the dependent measure. When appropriate, all ANOVAs were followed by Fisher Least Significant Difference post-hoc comparisons. In some cases (e.g. for the MOB and AOB), repeated-measures ANOVAs were used and subsequently followed by post-hoc analyses that were appropriate for repeated dependent measures (Honest Significant Differences). Only effects detected by the ANOVAs with a P-value lower than 0.05 are mentioned as significant in the Results.

Effect of urine exposure on Fos expression in the MOB and AOB

Three-way ANOVAs with sex, genotype and type of urine exposure as independent factors were performed on the number of Fos-ir granular cells in the ventral and dorsal MOB with the latter as repeated measure. The same analysis was used for statistical analysis of the number of Fos-ir cells in the mitral and granular cell layers (with layers as repeated measures) of the AOB.

Sex differences in brain Fos responses

Data from WT groups were analysed by two-way ANOVA with sex and type of urine exposure as independent factors. By doing this, we were

able to detect sex differences in Fos responses following exposure to urinary odours.

Genotype differences in brain Fos responses

Two-way ANOVAs with genotype and type of urine exposure as independent factors on Fos expression were carried out for each brain region described above. These analyses were performed for each sex separately.

Results

Effect of urine exposure on Fos expression in the MOB and AOB

MOB

Exposure to urine derived from an intact male or oestrous female induced significantly more Fos-ir cells in the MOB than exposure to water and this Fos response was very similar between WT and ArKO mice of both sexes (Table 1). Furthermore, this activation seemed to be higher in the dorsal (Fig. 1) than in the ventral part of the granular layer of the MOB. A three-way ANOVA on the number of Fos-ir cells in the dorsal and ventral parts of the granular cell layer of the MOB showed a significant effect of type of urine ($F_{2,55} = 6.13$, $P = 0.004$) and a significant difference between the dorsal and ventral part of the MOB (region, $F_{1,55} = 13.85$, $P = 0.0004$). Post-hoc analysis revealed that Fos responses in the MOB were similar whether urinary odours were derived from either an intact male or an oestrous female (male vs. female urine, $P = 0.44$). There was an almost significant trend for an interaction between region (dorsal vs. ventral) and type of urine ($F_{2,55} = 3.12$, $P = 0.052$), indicating that Fos expression was higher in the dorsal part of the MOB following exposure to opposite-sex urine (Fig. 1).

AOB

Exposure to male or oestrous female urine clearly induced Fos expression in both the granular and mitral cell layers of the AOB. This induction seemed to be quite similar in males and females and in ArKO and WT mice (Table 1). Repeated-measures ANOVA on the number of Fos-ir cells in the AOB revealed a significant effect of urine exposure ($F_{2,55} = 18.76$, $P < 0.0001$) and a significant sex × genotype × cell layer interaction ($F_{1,55} = 8.76$, $P = 0.004$). Post-hoc analysis of the urine effect indicated that urine exposure, independent of whether it was male or oestrous female urine, induced more Fos in the mitral and granular cell layers of the AOB than water. Furthermore, oestrous female urine induced more Fos-ir cells in the AOB than intact male urine in male and female mice (male vs. female urine, $P = 0.03$). Post-hoc analysis of the interaction showed that Fos expression was more prominent in the mitral than in the granular cell layer in WT males, whereas this was not the case in WT females (Fig. 2). This sex difference was not observed in ArKO mice with a tendency to show the reverse pattern of Fos expression (i.e. a higher Fos expression in the mitral cell layer than the granular cell layer only in ArKO females; mitral vs. granular layer in ArKO females, $P = 0.07$).

Sex differences in brain Fos responses

Overall, in WT females, exposure to opposite-sex urine induced a significant Fos expression in several brain regions receiving inputs from the olfactory bulbs, including some parts of the amygdala (MeA, MePD, MePV and ACo), MPoC and the VMH. These brain Fos responses to intact male urine were sexually dimorphic as they were absent (MeA, MePV, ACo, MPoC and VMH) or less clear (MePD) in WT males (Fig. 3). By contrast, exposure to oestrous female urine (as well as exposure to water) did not induce any significant Fos responses within these brain regions in male and female WT mice.

This was confirmed by two-way ANOVA revealing that exposure to intact male urine induced significantly more Fos expression than oestrous female urine or water in the ACo ($F_{2,29} = 6.756$, $P = 0.004$), MeA ($F_{2,29} = 5.744$, $P = 0.008$), MePD ($F_{2,29} = 16.493$, $P < 0.001$), MePV ($F_{2,29} = 23.245$, $P < 0.001$), MPoC ($F_{2,29} = 15.903$, $P < 0.001$) and VMH ($F_{2,29} = 6.946$, $P = 0.003$). Furthermore, statistical analysis confirmed the sex differences in Fos expression in response to male urine in several brain regions, including the ACo, MeA, MePV, MPoC and VMH, in WT mice with females showing a significant Fos response, whereas WT males did not. Thus, two-way ANOVA revealed significant sex × urine exposure interactions in WT mice for the ACo ($F_{2,29} = 4.579$, $P = 0.019$), MeA ($F_{2,29} = 7.821$, $P = 0.002$), MePV ($F_{2,29} = 8.804$, $P = 0.001$), MPoC ($F_{2,29} = 11.548$, $P < 0.001$) and VMH ($F_{2,29} =$

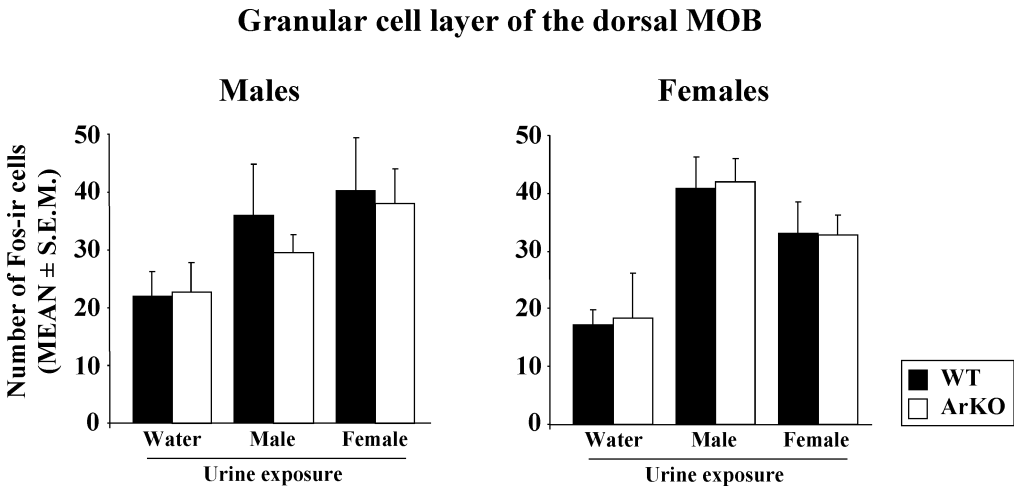

FIG. 1. Number of Fos-ir cells (mean ± SEM) in the granular cell layer of the dorsal MOB of WT and ArKO mice of both sexes following exposure to urinary odourants.

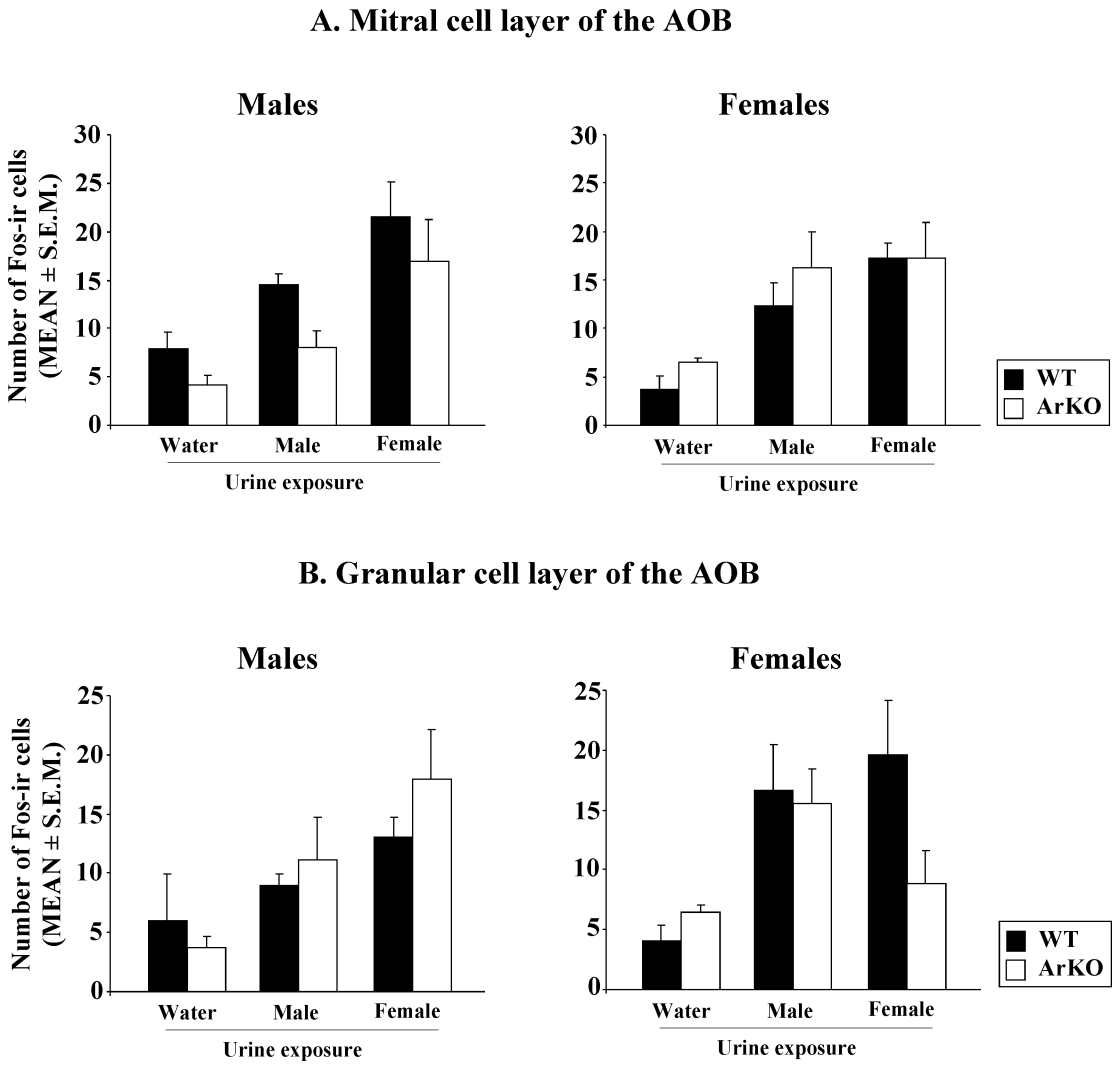

FIG. 2. Number of Fos-ir cells (mean ± SEM) in the mitral (A) and granular (B) cell layers of the AOB of WT and ArKO mice of both sexes following exposure to urinary odourants.

4.314, $P = 0.023$). Post-hoc analysis showed that exposure to intact male urine induced more Fos expression compared with control stimulation in WT females, whereas it did not in males (water vs. intact male urine: WT females, $P < 0.001$ for all of these brain regions; WT males, $P > 0.1$ for all of these brain regions). The effect of sex was also statistically significant in WT mice for the MPoC ($F_{1,29} = 9.951$, $P = 0.004$).

Genotype differences in brain Fos responses

Females

Exposure to intact male urine but not oestrous female urine induced significant Fos responses in the amygdala of both WT and ArKO females. Indeed, the effect of type of urine was significant in the four amygdaloid nuclei quantified: the ACo ($F_{2,26} = 5.705$, $P = 0.008$), MeA ($F_{2,26} = 11.452$, $P < 0.001$), MePD ($F_{2,26} = 14.282$, $P < 0.001$) and MePV ($F_{2,26} = 19.672$, $P < 0.001$). Fos expression in response to male urine was somewhat decreased in ArKO females as shown by the genotype × urine exposure interaction within the MePV ($F_{2,26} = 3.571$, $P = 0.043$), which was confirmed by post-hoc analysis showing that Fos responses to intact male urine were significant in the MePV of WT females (water vs. intact male urine exposure, $P < 0.001$) but less so in ArKO females (water vs. intact male urine exposure, $P = 0.056$; Fig. 3).

Significant Fos responses to male urine were also observed more centrally in the MPoC and VMH of WT females. Interestingly, ArKO females showed a significant increase in Fos expression in the MPoC but not in the VMH when exposed to male urine (Fig. 4). Indeed, the effect of type of urine was significant in these two brain areas (MPoC, $F_{2,26} = 18.244$, $P < 0.001$; VMH, $F_{2,26} = 8.612$, $P = 0.001$) but the effect of genotype ($F_{1,26} = 10.17$, $P = 0.004$) and the genotype × urine exposure interaction were only significant for the VMH ($F_{2,26} = 4.439$, $P = 0.022$). Post-hoc analysis revealed that exposure to intact male urine induced a significant Fos expression in the VMH of WT females, whereas it did not in ArKO females (water vs. male urine: in WT, $P = 0.00013$; in ArKO, $P = 0.32$).

Males

Exposure to urine derived from intact males or oestrous females did not induce any significant Fos responses in brain areas receiving

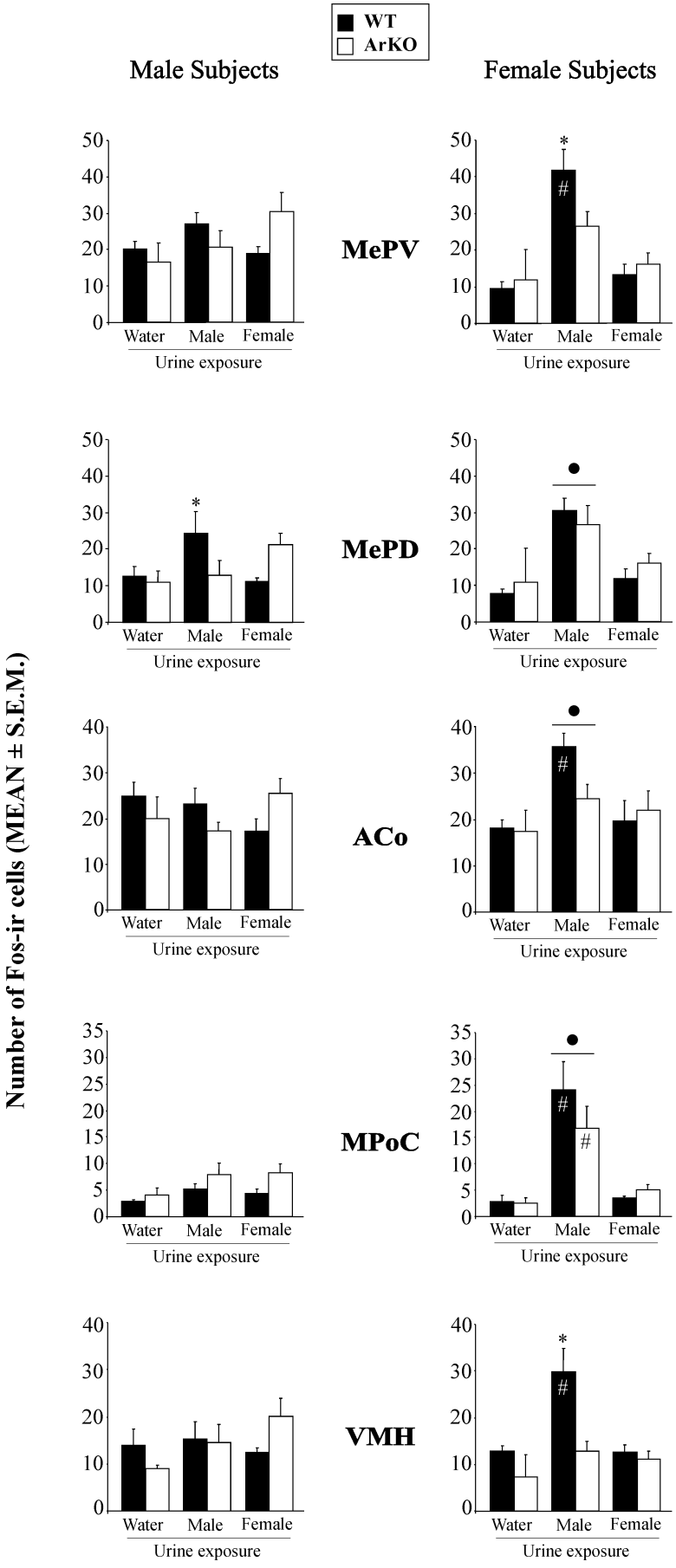

FIG. 3. Number of Fos-ir cells (mean ± SEM) in some brain regions, which are part of the olfactory pathways, in male (left panel) and female (right panel) WT and ArKO mice exposed to either water or urinary odours coming from an intact male or an oestrous female. $^{\bullet}P < 0.05$ different from females that were exposed to water or to oestrous female urine (post-hoc analysis on the effect of type of urine exposure). $^{*}P < 0.05$ different from females of the same genotype that were exposed to the other olfactory stimuli (post-hoc analysis on the genotype × urine exposure interaction). $^{\#}P < 0.05$ different from males of the same genotype exposed to intact male urine (sex difference in response to intact male urine).

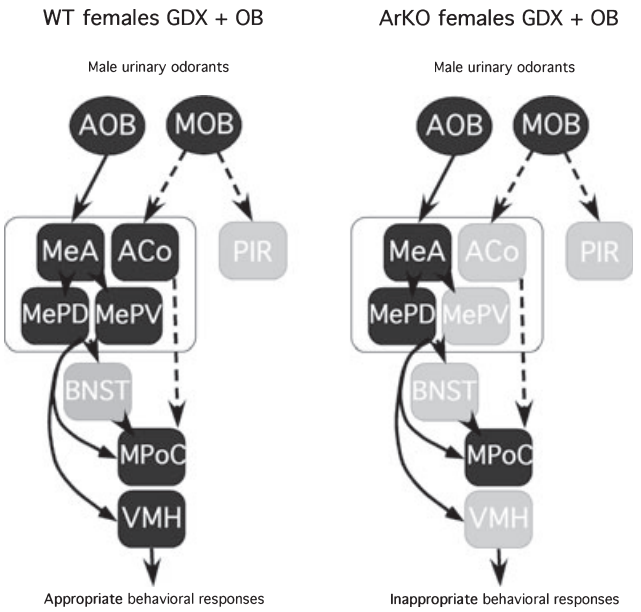

FIG. 4. Graphic representation of the brain structures of the main (dashed lines) and accessory (solid lines) olfactory pathways activated following male urinary odour exposure in WT and ArKO females. Regions in dark grey showed increased Fos expression following male odour exposure (compared with control stimulation), whereas areas in light grey did not.

afferents from the olfactory bulbs in either WT or ArKO males, with one exception, the MePD (Fig. 3). Indeed, two-way ANOVA on the number of Fos-ir cells quantified in the different brain regions did not reveal any significant effects except for the MePD, for which a significant genotype × urine exposure interaction was observed ($F_{2,29} = 4.26$, $P = 0.024$). Post-hoc analysis showed that exposure to intact male urine induced more Fos expression than exposure to water in the MePD of WT males ($P = 0.04$) but not of ArKO males ($P = 0.73$). In fact, Fos responses in the MePD of ArKO males were mainly observed (although not clearly significant) following exposure to urine derived from oestrous females (MePD, water vs. oestrous female urine exposure, $P = 0.058$ in ArKO and $P = 0.79$ in WT males). Interestingly, this trend to show Fos responses to oestrous female urine in ArKO but not WT males was also present in other parts of the amygdala (genotype × urine exposure interaction: ACo, $F_{2,29} = 2.888$, $P = 0.072$; MePV, $F_{2,29} = 2.79$, $P = 0.078$).

Discussion

The present study shows that the central processing of male-derived odours was affected in female ArKO mice, suggesting that the previously observed decrease in sexual behaviour may be due to an inability to respond correctly to odours derived from a potential mate. These results thus indicate that perinatal exposure to oestrogens may be necessary for processing of olfactory cues by the main and accessory olfactory systems in female mice. ArKO females that were treated with OB in adulthood to correct for their oestrogen deficiency showed a normal, female-typical, Fos activation at the level of the olfactory bulbs when exposed to male urinary odours. However, at more central levels, Fos responses were affected. Male odours still induced a significant Fos expression in the amygdala of ArKO females, although this increase was clearly reduced in comparison with WT female mice, but failed to activate the VMH, a brain region critical for the expression of reproductive behaviour in female mice (Fig. 4). We also observed different patterns of Fos expression following exposure to urinary odours from conspecifics of both sexes in the amygdala of WT and ArKO males, a region that seems to be essential for the integration of olfactory stimuli in mice (Choi *et al.*, 2005), suggesting that perinatal oestrogens may be involved in the development of this brain area in both males and females. Finally, we confirmed previously observed sex differences in neural activation of the accessory olfactory pathway following exposure to male odours (Halem *et al.*, 1999), although no significant Fos responses were observed in central brain regions of male ArKO mice when exposed to male urine, suggesting that these sex differences probably reflect the perinatal actions of androgens as was recently suggested by the work of Bodo & Rissman (2007).

The present results are in line with those obtained in our previous behavioural studies showing that the perception of sexual odourants *per se* is not affected in ArKO mice (Pierman *et al.*, 2006a; Wesson *et al.*, 2006). Overall, exposure to intact male or oestrous female urine induced significant increases in Fos expression at the level of the MOB and AOB in both WT and ArKO mice. Moreover, no sex differences were observed between males and females in their Fos responses in the olfactory bulbs to either male- or female-derived urine. Indeed, both male and female mice showed a significant Fos induction following exposure to male-derived urine as was also shown by Halem *et al.* (1999) and, as reported earlier by the same authors (Halem *et al.*, 2001), male and female mice treated with OB in adulthood showed equivalent activation of the AOB by odours derived from oestrous females. Taken together, these results suggest that processing of sexually relevant odours by the olfactory bulbs is not affected by the hormonal status of the respondent (as was also shown by Pfaff & Pfaffmann, 1969) and thus that the olfactory bulbs are probably not a critical target of oestrogen action during perinatal development.

Male-derived urine, but not oestrous female-derived urine, induced a significant Fos expression in WT females in central brain regions, which receive inputs from the MOB and AOB and which are critical for female reproductive behaviour and neuroendocrine function. This was particularly true for the medial amygdala, MPoC and VMH. Although classically the VMH is not seen as part of the olfactory systems, it is a target of olfactory inputs and plays an important role in female reproductive behaviour including olfactory preferences. For example, Robarts & Baum (2007) have shown that VMH lesions disrupt olfactory mate recognition and sexual receptivity in female ferrets. Furthermore, Choi *et al.* (2005) showed that a neural circuit delineated by the transcription factor Lhx6 conveys olfactory inputs of reproductive significance to the VMH. Interestingly, neural activation in these brain areas following exposure to urine of a potential mate was reduced in ArKO females. This reduction in neural Fos responses was already present at the level of the MePV and was more evident in the VMH. The decreased activation of the amygdala is interesting as it is well known that, in mice, defensive and reproductive values of olfactory stimuli are integrated within this brain area (Choi *et al.*, 2005). A representation of the stimulus reaches the medial pre-optic area and the VMH where it leads to appropriate hormonal and behavioural changes, e.g. facilitating sexual receptivity in female mice. These results of a decreased neuronal activation of the VMH are particularly interesting with regard to our earlier observation of a reduction in lordosis behaviour in ArKO females (Bakker *et al.*, 2002a). The present results thus suggest that, in ArKO females, olfactory cues from a potential mate are correctly detected at the level of the olfactory bulb but that the integration of their reproductive value at the level of the amygdala and VMH is affected, which may explain their deficits in the expression of sexual behaviour.

TABLE 1. Mean (± SEM) number of Fos-ir cells in the granular cell layer of the dorsal and ventral MOB, and in the mitral and granular cell layers of the AOB in males and females of both genotypes when exposed to urinary odourants from conspecifics

	Genotype					
	WT			ArKO		
	Urine exposure					
	Control	Intact male	Oestrous female	Control	Intact male	Oestrous female
Male subjects						
MOB						
Dorsal	22 ± 6	36 ± 5.6	40.2 ± 5.6	23 ± 5.6	29.7 ± 5.6	38.3 ± 5.6
Ventral	15.6 ± 6	26.3 ± 5.5	27.2 ± 5.5	19 ± 5.5	25.3 ± 5.5	26.3 ± 5.5
AOB						
Mitral	7.9 ± 2.9	14.5 ± 2.6	21.6 ± 2.6	4.2 ± 2.6	8.1 ± 2.6	16.9 ± 2.6
Granular	6 ± 3.3	9 ± 3	13 ± 3	3.7 ± 3	11.2 ± 2.9	17.9 ± 3
Female subjects						
MOB						
Dorsal	17.3 ± 5.6	40.8 ± 5.5	33 ± 5.6	18.5 ± 9.7	41.8 ± 5.6	32.8 ± 5.6
Ventral	20.7 ± 5.5	35.8 ± 5.6	25.5 ± 5.5	24.5 ± 9.5	29.5 ± 5.5	20.7 ± 5.5
AOB						
Mitral	3.8 ± 2.6	12.3 ± 2.6	17.3 ± 2.6	6.5 ± 4.5	16.3 ± 2.6	17.3 ± 2.6
Granular	4.1 ± 3	16.7 ± 2.9	19.6 ± 3	6.5 ± 5.2	15.5 ± 3	8.8 ± 3

In WT males, very weak Fos responses were observed in central brain regions after exposure to either male or oestrous female urinary odours. However, in a pilot study (data not shown) we found that exposure to bedding soiled by oestrous females was able to induce significant Fos responses within the MePD and medial pre-optic area of castrated, OB-treated WT males (as was previously shown in intact males by Aste *et al.*, 2003) but, rather surprisingly, exposure to urine derived from oestrous females failed to induce any Fos responses in these males. The present study confirmed those results obtained in our pilot study, suggesting that the Fos responses observed in these brain areas of WT males exposed to oestrous female bedding were not induced by the urinary odour cues contained in the bedding. However, it should be noted that male subjects in the present study were sexually naive and it is possible that sexual experience with a receptive female may influence the induction of neural activation of central brain regions following exposure to urinary odours of a potential mate as has been shown in male rats (Hosokawa & Chiba, 2005).

Although Fos responses were less robust in central brain regions of male mice, comparison of WT and ArKO males showed some interesting findings. Indeed, statistical analysis of Fos responses in the MePD revealed that only WT males showed a significant Fos response in this brain region following exposure to male urine, whereas ArKO males only showed a significant Fos response when exposed to female urinary odours (this was also present at the level of the ACo and MePV). It should be noted that, even though sexual behaviours are largely corrected in male ArKO mice when treated with OB in adulthood (using the same treatment regimen as in the present study), their odour preferences are clearly not, i.e. OB-treated male ArKO mice failed to show a preference for oestrous female odours in a Y-maze and still investigated significantly less body odours from conspecifics (Bakker *et al.*, 2004). Furthermore, the presence of a potential mate was not sufficiently rewarding to induce a conditioned place preference in ArKO males, whereas it was in WT males (Pierman *et al.*, 2006b). Therefore, the present study suggests some changes in the integration/interpretation of olfactory stimuli in the amygdala of ArKO males that could potentially contribute to the absence of olfactory preferences in these animals.

Finally, the present study confirms the presence of sexually dimorphic Fos responses in the accessory olfactory projection pathways to male-derived urinary odours in WT mice. Sex differences were not present at the peripheral levels (i.e. the MOB and AOB) but emerged at the level of the medial amygdala (MeA and MePV) and the ACo where neuronal Fos expression induced by male odours was higher in female than male mice. At more central levels, i.e. the medial pre-optic area and ventromedial hypothalamus, only female mice showed a significant Fos response following exposure to male urinary odours. These sex differences in central Fos responses to male urinary odours were less obvious in ArKO mice. They were dramatically reduced within the amygdala, suggesting that processing of sexually relevant odours is affected, at least partially, by perinatal actions of oestrogens. However, male ArKO mice did not show a Fos response to male odours in central brain regions, such as the MPoC, as was previously observed in male rats that were neonatally treated with the aromatase inhibitor 1,4,6-androstatrien-3,17-dione (Bakker *et al.*, 1996). These results thus suggest that, in contrast to the male rat, the sexual differentiation of the neural Fos responses to male odours may not reflect perinatal actions of oestrogens in the male mouse nervous system. These results are in line with the recent data published by Bodo & Rissman (2007). In this experiment, castrated OB-treated male mice carrying the testicular feminization mutation of the androgen receptor showed an increase in Fos expression within the bed nucleus of the stria terminalis and the medial pre-optic area following exposure to intact male bedding. This Fos response was present in testicular feminization males and WT females but not in males, suggesting that sexual differentiation of neural responses in the medial pre-optic area to opposite-sex odours depends on androgens in males.

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Abbreviations

ACo, anterior cortical amygdaloid nucleus; AOB, accessory olfactory bulb; ArKO, aromatase knockout; ir, immunoreactive; MeA, anterior medial amygdala; MePD, posterodorsal part of the medial amygdala; MePV, posteroventral part of the medial amygdala; MOB, main olfactory bulb; MPoC, central part of the medial pre-optic nucleus; NGS, normal goat serum; OB, oestradiol benzoate; VMH, ventromedial hypothalamic nucleus; WT, wild-type.

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