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Masculinization induced by neonatal exposure to PGE₂ or estradiol alters c-fos induction by estrous odors in adult rats

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Abstract

Processing of relevant olfactory and pheromonal cues has long been known as an important process necessary for social and sexual behavior in rodents. Several nuclei that receive input from the vomeronasal projection pathway are involved in sexual behavior and show changes in immediate early gene expression after stimulation with a variety of sex-related stimuli. The nuclei in this pathway are sexually dimorphic due to the early patterning events induced by estradiol derived from testicular androgens, which developmentally defeminize and masculinize the brain and adult sexual behavior. Masculinization can be induced independently of estradiol via prostaglandin-E₂ (PGE₂), and therefore assessed separately from defeminization. Here we examined the effects of brain defeminization and masculinization on neuronal response to sex-related odors using Fos, the protein product of the immediate early gene *c-fos*, as an indicator of activity. Female rat pups treated with a cyclooxygenase-2 inhibitor, to reduce PGE₂, plus estradiol, estradiol alone, and PGE₂ alone were exposed to estrous female odor as adults and the resulting Fos expression was examined in the medial amygdala, preoptic area, and ventromedial nucleus of the hypothalamus. Defeminized and/or masculinized females all showed patterns of Fos activity similar to control males and significantly different from control females. These results suggest that early exposure to estradiol and PGE₂ do not affect olfaction in females, but switch the activity pattern of sex-related nuclei in females to resemble that of males following exposure to sexually-relevant cues.

Keywords

estradiol; prostaglandin-E₂; Fos; sexual behavior; pheromones

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1. Introduction

The ability of mammals to exhibit a sexually dimorphic repertoire of reproductive behaviors is the result of early neural organizational events that are dictated by steroid hormones, in particular testosterone and its metabolites, estradiol and dihydrotestosterone. The neonatal rodent brain's default phenotype is female, however early exposure to hormones results in both masculinization, the ability to display male sex behavior in adulthood, and defeminization, a separate process that removes the ability to express female sex behavior [reviewed in ^[1]]. During a critical perinatal period, hormone-sensitive brain regions are sculpted via exposure to estradiol, which is aromatized in the brain from testosterone produced in the developing testes. Brain organization during this period permanently modifies neural structures, resulting in sex-specific neuroendocrine physiology and behavior (reviewed in [¹] and [²]). Accordingly, defeminization and masculinization of the brain can be experimentally induced in newborn females by mimicking the male hormonal profile with injections of testosterone or estradiol [³].

In adulthood, olfaction, a dominant sense in rodents, plays a large role in sexual receptivity [^{4,5}]. Neurons within the vomeronasal projection pathway are activated by pheromonal cues from conspecifics, and relay information necessary for mate selection [⁶]. Pheromonal signals are detected by the vomeronasal organ (VNO), sent through the accessory olfactory bulb, and then relayed through the subdivisions of the medial amygdala (MeA) to the bed nucleus of the stria terminalis (BNST), the preoptic area (POA), and the ventromedial nucleus of the hypothalamus (VMN) [^{7,8}]. These nuclei all have high levels of steroid hormone receptors [⁹] and serve as integration sites where external information from pheromones and hormonal stimulation interact to affect behavior [^{10,11}]. The VNO and downstream nuclei in the vomeronasal projection pathway are sexually dimorphic [^{12, 13}] due to the masculinizing effects of neonatal estrogen exposure. The brain areas associated with the pathway, such as the POA, MeA and VMN, are also larger in males [^{14, 15, 16, 17, 18}].

In addition to influencing the volume of sex-related nuclei in the vomeronasal projection pathway, early steroid hormone exposure establishes sexually dimorphic synaptic patterning in these areas [^{19, 20, 21}]. For example, in the male POA estradiol up-regulates cyclooxygenase-2 (COX-2), resulting in an increase in prostaglandin-E₂ (PGE₂), which stimulates dendritic spine proliferation through AMPA-kainate glutamate receptor activation [²²]. This organizational event is essential for the expression of adult male sex behavior. Blocking the production of PGE₂ in newborn male rats with the COX inhibitor indomethacin, or in utero exposure to aspirin, prevents normal synaptic patterning in the POA and disrupts sexual behavior in adulthood, such that males no longer attempt to mount receptive females [²³]. Similarly, administration of PGE₂ to newborn female rats masculinizes the POA, so that adult females primed with testosterone display high rates of male sex behavior when presented with a receptive female [²³]. Thus, the actions of PGE₂ in the developing brain are both necessary and sufficient for masculinization of sex behavior. However, we have also determined that PGE₂ action early in life has no effect on the separate, hormone-dependent process of defeminization [²⁴]; a neonatal female treated with PGE₂ alone will exhibit either male or female sexual behavior as an adult when provided the appropriate hormonal milieu. Conversely, a male pup treated with a prostaglandin synthesis inhibitor will display neither male nor female sexual behavior due to prevention of masculinization but uninterrupted defeminization. The direct actions of PGE₂ on other brain areas in the vomeronasal projection pathway are relatively unknown.

Various techniques have been applied to examine the involvement of the vomeronasal projection pathway in sexual behavior, including targeted lesions, hormonal manipulations, electrical stimulation [^{25, 26}], and more recently immediate early gene (IEG) activation as a

marker of signal transduction activity. IEG expression can be the result of neuronal activation or inhibition [27, 28]. Visualization of IEGs can confirm activity in many nuclei at once after a single experimental exposure, which allows for delineation of neural pathways. Immunoreactivity (IR) of Fos protein, the product of the gene *c-fos*, has been a particularly popular IEG used in visualizing neuronal activation associated with sexual stimuli [reviewed in 29]. For example, in male and female rats, Fos IR has been observed in many hormone-concentrating brain areas associated with sexual behavior, including the MeA, POA, and VMN among other areas. In both sexes, Fos is expressed in the MeA and POA in response to female scent on soiled bedding [30]. In sexually experienced male rats, it has been demonstrated that volatile odors from an inaccessible estrous female also stimulate Fos expression in these nuclei [31,32]. Fos IR induced by estrous odors is amplified in both sexes following testosterone treatment [33]. The scent of a male conspecific also increases Fos IR in the MeA, POA and VMN of hormone-primed female rats [34, 35] and ferrets [36]. Increasing the amount of sexual stimulation of male or female rats induces higher levels of Fos expression in nuclei associated with sexual behavior, with the highest count of Fos-positive cells observed following ejaculation [37, 38, 39, 40].

The ability of activational hormonal effects to modulate or amplify the induction of Fos IR following olfactory or sexual stimuli is well established and the organizational action of gonadal steroids during the perinatal sensitive period is equally important. Blocking developmental masculinization of the male brain with an aromatase inhibitor results in Fos expression and sexual behavior similar to females after exposure to male odor [41, 42]. In our previous investigations of the role of PGE₂ in masculinization of sexual behavior we have found that animals, in which prostaglandin synthesis was blocked neonatally, will investigate an estrous female but will not attempt to mount. This led us to hypothesize that processing of olfactory input may be altered in these animals. We also predicted that masculinized and defeminized females would display patterns of neural activation in response to estrous odors similar to males. Therefore, the current study examined Fos expression after non-contact exposure to an estrous female in males and females developmentally manipulated to induce defeminization and/or masculinization via the prostaglandin system.

2. Methods

2.1 Subjects

Four timed-pregnant Sprague-Dawley rats purchased from Harlan Laboratories (Fredrick, MD) were housed on a 12-h reverse light/dark cycle and allowed *ad libitum* access to food and water. On the day of birth (postnatal day 0), pups were randomized across litters and assigned to experimental groups. Animal care and experimental treatments were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore, MD, USA.

2.2 Experimental design

2.2.1 Developmental Manipulations—On postnatal (PN) day 0 and PN1, pups received bilateral intracerebroventricular (ICV) infusions of either 1.25 µg PGE₂ per hemisphere or phosphate buffered saline (PBS). Pups were cold anesthetized and bilateral injections were performed by hand using Bregma as a landmark to locate the lateral ventricle. A 23 gauge, 1µl Hamilton syringe was used to deliver a 1 µl infusion volume over 60 seconds at a depth of 1.5 mm below the skull. Each pup also received 0.1 ml subcutaneous (s.c.) injections 17β-estradiol 3-benzoate (EB, 100 µg), EB (100 µg) and the cyclooxygenase inhibitor, indomethacin (50 µg), or sesame oil vehicle. The doses of PGE₂, indomethacin, and EB are the same as those used previously and proven effective [23].

Animals were divided into the following treatment groups: 1) Control males (N = 6; received ICV PBS, and oil s.c.), 2) masculinized females (N = 6; ICV PGE₂, oil s.c.), 3) masculinized and defeminized females (N = 6; ICV PBS, EB s.c.), 4) defeminized females (N = 5; ICV PBS, EB and indomethacin s.c.), and 5) control females (N = 6; ICV PBS, oil s.c.).

On PN20 animals were weaned and sorted into cages based on their experimental group. On PN48 through 51, all animals were gonadectomized under ketamine/acepromazine (75 mg/2.5 mg per kg) anesthesia and implanted with silastic capsules (3 cm long, 1.57 mm inner diameter, 3.18 mm outer diameter) containing testosterone propionate (TP), to mimic the hormone profile of an adult male. Stimulus females, which were untreated on PN0, were also ovariectomized on PN48-51.

2.2.2 Female exposure—Animals were numbered prior to behavior by experimenters blind to treatment. All behavior tests were performed during the dark segment of the animals' light cycle under red light illumination. Approximately three weeks after surgery and TP capsule implantation (around PN72), animals were acclimated to the exposure arena (tinted plexiglass, 49 cm L, 37 cm W, 24 cm H, divided in half with a wire mesh screen). Acclimation was given 15 minutes a day for 3 days before the test day.

Stimulus females were primed for behavioral estrous with 10 µg EB (s.c., in oil) on two consecutive days followed by 1 mg progesterone (s.c., in oil) 4 hours prior to testing. This hormonal regime reliably produces a high level of sexual receptivity [23].

The sexually naïve experimental animals were placed into the empty arenas for 15 minutes, then a sexually-receptive stimulus female was placed in the arena behind a wire mesh screen for an additional 15 minutes. Thirty minutes later, the experimental animals were overdosed with Nembutal (IP, ~ 0.15 ml/100g body weight), and transcardially perfused with cold saline plus 2% sodium nitrite followed by 4% paraformaldehyde plus 2.5% acrolein. Brains were stored in 4% paraformaldehyde overnight, then switched to 30% sucrose.

2.3 Tissue preparation and Fos ICC

Using a cryostat, brains were cut into 30 µm coronal sections. Every sixth section was collected to form 6 sets. Two sets of approximately 25 free-floating sections each were processed for Fos IR. Immunocytochemistry was performed as described in [43]. Briefly, sections were rinsed of cryoprotectant with 0.1M PBS, incubated in 0.01% sodium borohydride, rinsed with PBS, and incubated in a primary rabbit polyclonal anti-Fos antibody (1:100,000; Oncogene, Cambridge, MA) for 48 hours at 4°C. After rinsing with PBS, sections were incubated in a biotin-conjugated anti-Rabbit IgG secondary antibody (1:600; Vector, Burlingame, CA) for one hour, rinsed again in PBS, and incubated in an Elite ABC kit (Vector) for an additional hour before being rinsed with 0.175 M sodium acetate, incubated in nickel II sulfate diaminbenzidine chromagen solution, and rinsed again with sodium acetate to stop the reaction. Sections were mounted onto gelatin-coated slides and coverslipped using Permount (Fisher Scientific Co., Pittsburgh, PA).

2.4 Data Analysis

Fos-positive cells, characterized by a darkly stained nucleus, were counted in the MeA, POA, VMN, and perirhinal cortex (PRC). Three to 5 sections from each hemisphere per animal per brain region were counted and averaged together to obtain a mean for each animal. A brain atlas was used to reference the location of regions of interest [44] (Figure 1A, Figure 2A, Figure 3A). The third ventricle was used as a landmark to capture the same area of the POA and VMN in each animal. Likewise, the optic tract was used to locate a standard field of view in the MeA. Nuclei were visualized under 10× magnification and all Fos-positive cells visible in a

standardized field of view (examples shown in Figure 1C–D, Figure 2C–D, Figure 3C–D) were captured using NIH image (version 1.63) and counted by hand. Mean and percent Fos data were analyzed by one-way ANOVA or Kruskal-Wallis tests respectively, followed by Mann Whitney U analysis when appropriate. All data analysis was performed using StatView (version 5.0) with an alpha level of 0.05 as a criterion for statistical significance.

3. Results

Separate ANOVAs of each brain region indicated no significant differences in mean Fos positive cell counts between groups (PRC, $F_{4,24} = 1.043$, $p = 0.406$; MeA, $F_{4,24} = 1.275$, $p = 0.307$; POA, $F_{4,22} = 1.777$, $p = 0.169$; VMN, $F_{4,24} = 1.139$, $p = 0.362$) due to a high degree of variation between animals in each group (stdev > 22% of mean). To reduce variability between individuals, we normalized the mean number of Fos positive cells measured in the MeA, POA and VMN to the mean number obtained in the PRC for each animal. F tests revealed the percentage of PRC Fos in the MeA and VMN had a normal distribution, whereas data obtained for the POA showed a significant departure from normality. In each brain area analyzed, there was a main effect of experimental group on percent Fos positive cells (MeA, ANOVA, $F_{4,24} = 4.568$, $p = 0.0069$; POA, Kruskal-Wallis, $p = 0.0039$; VMN, ANOVA, $F_{4,24} = 2.295$, $p = 0.0092$). For ease of evaluation, the percentage of cells expressing Fos in the control female group was standardized to 100% and compared to each experimental group. In a separate experiment using the exact experimental conditions described above, an additional group of male and female rats were exposed to the behavioral arena in the absence of an estrous female. In each brain region counted, there were no differential effects of sex on Fos IR when animals were placed in the testing arena without an estrous female present (Table 1).

3.1 MeA

In the MeA, Kruskal-Wallis test indicated a significant main effect of experimental group on percent Fos-positive cells ($p = 0.0019$). Individual Mann Whitney U tests revealed that there was a significantly smaller percentage of Fos positive cells in control males ($p = 0.0039$), masculinized females ($p = 0.0039$), masculinized/defeminized females ($p = 0.0039$), and defeminized females ($p = 0.0062$) compared to control females (Figure 1B). These data indicate that developmental masculinization and defeminization of the female rat brain with PGE₂ or estradiol results in a pattern of neuronal activation in response to sexual stimuli similar to normal males.

3.2 POA

Kruskal-Wallis test also indicated a main effect of treatment group on the percentage of Fos IR in the POA ($p = 0.0011$). Control males had the lowest percentage of Fos-positive cells in the POA ($45.3\% \pm 8.7\%$), which was about half that of control females ($p = 0.009$). Masculinized ($p = 0.0106$), masculinized/defeminized ($p = 0.0285$), and defeminized ($p = 0.0090$) females also had a significantly lower percent of Fos-positive cells in the POA after estrous female exposure (Figure 2B) than control females. These results suggest that after pheromonal stimulation, developmentally manipulated female rodents have a pattern of POA engagement similar to males.

3.3 VMN

Like the MeA and POA, Kruskal-Wallis test also showed a main effect of treatment group on the percentage of Fos-positive cells in the VMN ($p = 0.0056$). Control females had a significantly higher percentage of Fos IR in the VMN compared to control males ($p = 0.0039$), PGE₂ treated females ($p = 0.0039$), EB treated females ($p = 0.0039$), and females given indomethacin and EB ($p = 0.0062$) (Figure 3B). As seen in the other brain areas examined,

these results suggest that developmental masculinization and defeminization of females results in a male pattern of Fos expression in the VMN.

4. Discussion

Based on our previous behavioral observations, we predicted that olfactory signals emanating from an estrous female would evoke a differential pattern of Fos IR in the brains of animals that were masculinized and defeminized neonatally (either by exogenous estrogen or endogenous signals) versus animals which were masculinized but not defeminized (PGE₂ alone) or defeminized but not masculinized (EB and indomethacin). However, contrary to our prediction we found that masculinized/defeminized, masculinized, and defeminized groups had similar numbers of Fos-positive cells in sex related nuclei; all groups had significantly less Fos-IR than control females. This means that developmental administration of PGE₂ and estradiol not only have the ability to change the size and synaptic patterning of nuclei, but also their neuronal activity in response to estrous odors. These results also demonstrate that developmental manipulations of females using PGE₂ and estradiol do not interrupt olfactory processing, since females masculinized by either estradiol or PGE₂ administration processed olfactory signals correctly and similarly to males. Previous work has shown that although nuclei of the vomeronasal projection pathway are sexually dimorphic, sex differences in neuron number do not account for differences in Fos expression between males and females in response to estrous odor [33]. The results from the present study suggest that control females have greater *c-fos* activation after non-contact exposure to an estrous female than sexually naive males, and defeminized or masculinized females.

Upon initial inspection of our results, the decrease in Fos induction in brain regions classically associated with male sex behavior (i.e. the MeA and POA) in males and masculinized females seems counterintuitive. However, the majority of studies showing increased Fos expression in the MeA and POA following estrous odor exposure have used male subjects with prior sexual experience [30, 31, 32, 33, 45] and early studies suggested that preference for estrous odor is dependent on sexual experience in males [46]. In contrast, females do not require prior sexual experience for Fos induction in response to [34,35] or preference for [47] sex-related odors. Since our animals did not receive sexual experience prior to estrous-odor exposure, it is not surprising that the increase in Fos expression in females would be greater than in males. We chose to use sexually naïve animals in the present study because females treated with indomethacin and EB will not mount another female. The difference in interactions with a sexually-receptive female would therefore be different between groups and present a possible confound.

Endogenous hormones in males, and exogenous hormone treatment of females, during development leads to an increase in dendritic spine number and spinophilin levels in the developing rat VMN [48, 24]. However, unlike in the POA, neonatal PGE₂ treatment does not effect spinophilin levels in the VMN, indicating that masculinization of this region has a mechanism distinct from the POA [24], which we have recently confirmed [49]. PGE₂ does not directly alter the morphology of the VMN, but in the present study we observed that neonatal treatment with PGE₂ changes the ability of estrous cues to induce Fos-IR in the VMN. In addition, we have not found any impact of neonatal PGE₂ treatment on the developing amygdale (unpublished observation), but the present data suggest that PGE₂-induced masculinization results in Fos expression similar to males. This suggests the possibility that upstream alterations induced by PGE₂ or estradiol treatment could be affecting the propagation of pheromonal signals through the vomeronasal projection pathway to the MeA and VMN. Olfactory signals from estrous females are transmitted through the nuclei of the vomeronasal projection pathway, initiating at the VNO and sequentially passing through the MeA, POA, and VMN. Therefore the possibility exists that PGE₂ and estradiol exert their masculinizing

effects on structures near the origin of the pathway, and altered signals from sex related odors are transmitted to nuclei further downstream. Changes in the activity of neurons in the beginning of the pathway in response to sex-related odors may alter the activity of downstream nuclei that have not been masculinized.

Neonatal prostaglandin-mediated masculinization and estradiol-induced masculinization/defeminization are essential for normal adult sexual behavior and synaptic patterning. The results from this study provide evidence that processing of odorant signals transmitting social and sexual information is a sexually dimorphic phenomenon also regulated by early exposure to PGE₂ and estradiol.

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Figure 1.

Fos IR in the MeA. A. Atlas image showing the location of Fos measurement in the MeA. Images were collected from the posterodorsal region of the MeA [44], indicated by the striped region. B. The percentage of Fos IR in males and developmentally manipulated females was significantly lower compared to control females (* $p < 0.01$ for each treatment group compared to control female). C., D. Representative fields counted for Fos expression at 10 $\times$ magnification in control males and females, respectively. Scale bar is equal to 100 μ m. Insets show location of representative fields at 4 $\times$ magnification.

Figure 2.

Fos IR in the POA. A. Atlas image showing the location of the POA [44], indicated by the striped region. B. The percentage of Fos IR in males and developmentally manipulated females was significantly lower compared to control females (* $p < 0.05$ for each treatment group compared to control female). C., D. Representative fields counted for Fos expression at 10 $\times$ magnification in control males and females, respectively. Scale bar is equal to 100 μ m. Insets show location of representative fields at 4 $\times$ magnification.

Figure 3.

Fos IR in the VMN. A. Atlas image showing the location of the VMN [44], indicated by the striped region. B. The percentage of Fos IR in males and developmentally manipulated females was significantly lower compared to control females (* $p < 0.01$ for each treatment group compared to control females). C., D. Representative fields counted for Fos expression at 10 $\times$ magnification in control males and females, respectively. Scale bar is equal to 100 μ m. Insets show location of representative fields at 4 $\times$ magnification.

Table 1

Fos IR normalized to perirhinal cortex in male and female rats in the absence of an estrous female. In each brain region analyzed, there were no significant differences in % PRC Fos between male and female control animals when placed into the behavioral arena without exposure to estrous female odor.

Fos IR Normalized to Perirhinal Cortex (expressed as %)			
	Female (n=6) (mean % +/- SEM)	Male (n=5) (mean % +/- SEM)	p value
MeA	279.98 +/- 79.56	253.16 +/- 46.13	0.387121945
POA	174.62 +/- 38.09	160.14 +/- 38.03	0.395521724
VMN	275.15 +/- 52.62	287.54 +/- 48.26	0.434738009