Research report

Fos immunoreactivity in the rat brain following consummatory elements of sexual behavior: a sex comparison

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Abstract

In the present study a comparison was made between the distribution of Fos immunoreactivity in the brain of female and male rats following successive elements of sexual behavior. The distribution of Fos immunoreactivity following either mounting, eight intromissions or one or two ejaculations was compared with that in control animals. In both females and males, Fos immunoreactivity was induced in the medial preoptic nucleus, posteromedial part of the bed nucleus of the stria terminalis, posterodorsal part of the medial amygdala, and the parvicellular part of the subparafascicular thalamic nucleus. In addition, Fos immunoreactivity in females was induced in the ventrolateral part and the most caudoventral part of the ventromedial nucleus of the hypothalamus and in the premammillary nucleus. Differences between females and males were detected in the phases of sexual activity that resulted in Fos immunoreactivity in these brain areas, allowing more insight in the nature of the sensory and hormonal stimuli leading to the induction of Fos immunoreactivity. The posteromedial bed nucleus of the stria terminalis appears to be involved in chemosensory investigation, while specific distinct subregions are only activated following ejaculation. In addition, the parvicellular subparafascicular nucleus and the lateral part of the posterodorsal medial amygdala appear to be involved in the integration of viscero-sensory input. The neural circuitries underlying sexual behavior in males and females appear to be similar in terms of integration of sensory information. In males the medial preoptic nucleus may be regarded as the brain area where the integration of sensory and hormonal stimulation leads to the onset of male sexual behavior, while in females the ventrolateral part of the ventromedial hypothalamic nucleus appears to have this function. In addition, Fos immunoreactivity was distributed in distinct clusters in subregions within various brain areas in males and females. This was observed especially in the posteromedial bed nucleus of the stria terminalis and posteriorodorsal medial amygdala, but also in the parvicellular subparafascicular nucleus, ventromedial hypothalamic nucleus and ventral premammillary nucleus. It appears that relatively small subunits within these nuclei seem to be concerned with the integration of sensory and hormonal information and may play a critical role in sexual behavior.

Keywords: Male rat; Female rat; Medial preoptic area; Bed nucleus of the stria terminalis; Medial amygdala; Midbrain; Steroids; Copulation

1. Introduction

The immunocytochemical visualization of the protein product (Fos) of the immediate early gene c-fos has been used as a marker for the activation of neurons stimulated in various ways [14,25]. Several investigators have studied neural activation in the male rodent brain following copulation using Fos immunoreactivity (IR). Increases in Fos-IR were reported in the medial preoptic area (MPOA), the bed nucleus of the stria terminalis (BNST), the medial amygdala (MEA), and in the midbrain central tegmental field in male rats [2,37,46] and hamsters [11,18,48].

In a previous study from our laboratory [6,7], the distributions of Fos-IR neurons were compared following different behavioral situations in which male rats were able to display consummatory aspects of sexual behavior, including intromissions and ejaculations, or appetitive aspects during the interaction with anestrous females or in a sex-odor rich environment. Differences in the distribution of Fos-IR after these different behavioral aspects of male copulatory behavior were observed in specific subregions of the MPOA, BNST, MEA, and the central tegmental field. Fos-IR in the medial preoptic nucleus (MPN) and in the parvicellular part of the subparafascicular thalamic nucleus (SPFp) was observed only following mating including intromissions and ejaculations, suggesting that these areas were mainly involved in the performance of mating. Fos expression in the posteromedial part of the
BNST (BNSTpm) and in the posterodorsal part of the MEA (MEApd) was evident in males following mating or chemosensory investigation. However, mating and not chemosensory investigation was followed by appearance of clusters of Fos-IR neurons in the BNSTpm and MEApd.

Until the present time, we have studied the distribution of Fos-IR following mating performance without taking in account the elements: mounts, intromissions and ejaculations separately. Therefore it was not clear which behavioral elements specifically resulted in Fos-IR in the particular brain areas. In the present study, we studied the distribution of Fos-IR neurons following each of these successive elements of the consummatory phase of sexual behavior to understand what specific stimuli from the complex behavioral situations result in increases in Fos-IR in the male rat brain.

The activation of Fos-IR following copulatory behavior has also been studied in female rodents. Several investigators have reported that Fos-IR is induced following mating in female rodents in the MPOA, BNST, MEA, central tegmental field, ventromedial hypothalamic nucleus (VMH), and periaqueductal gray (PAG) [10,21,33,34,38,42,46]. It was also reported that vaginocervical stimulation, by intromissions and ejaculations of the male partner or by manual probing, was followed by a much stronger induction of Fos-IR than the Fos-IR after lordosis behavior induced by flank stimulation [33,38,42]. Since vaginocervical stimulation seems to be essential for the induction of Fos-IR in the female brain, we were interested to know which phases of sexual behavior induce Fos-IR in specific parts of the brain. Therefore, in addition to investigating the distribution of Fos-IR in the male rat brain following several phases of sexual behavior, the distribution of Fos-IR in the brain of their female partners was studied to determine if the distribution of Fos-IR neurons after various phases of sexual behavior differs between males and females.

The induction of Fos-IR was studied in sexually experienced male and female rats, following mounting behavior, intromissions and either one or two successive ejaculations. In addition to a quantitative analysis of the numbers of Fos-IR neurons in the MPN, BNSTpm, MEApd and SPFp, the distribution of the Fos positive neurons was investigated.

2. Materials and methods

2.1. Animals

Male and female Wistar rats (n = 15 each, 3 months of age) obtained from the local breeding facilities, were group-housed by sex in separate artificially lighted rooms on a reversed 12:12 light/dark cycle. Lights in the female colony were off between 12.00 and 24.00 h; lights in the male colony were off between 05.00 and 17.00 h. Food and water were available at all times. Two days before testing, rats were housed individually.

2.2. Experimental protocols

All male and female rats were sexually experienced. Male rats were allowed to copulate during four or five pre-test mating sessions and were included after displaying ejaculation within the first 10 min of the last two mating sessions. The female rats were bilaterally ovariectomized three weeks prior to testing. Sexual receptivity was induced using a standard regimen of exogenous estrogen and progesterone. This includes administration of 50 μg estradiol benzoate (EB)/0.1 ml arachidis-oil 48 h before testing, and 500 μg progesterone (P)/0.1 ml arachidis oil 4 h before testing. Females received hormone treatment and mating experience with different sexually experienced male partners, one week before testing.

All testing occurred during the second half of the male’s dark period (between 12.30 and 13.15 h) when the males show vigorous sexual behavior. By contrast the female receptive period begins at the onset of the dark period. To maximize sexual behavior in males and females, they were housed under different light/dark periods. Testing was performed under dim red light and at no time were animals exposed to bright white light to avoid Fos expression induced by light.

To study the induction of Fos-IR in different brain areas in males and females after several phases of sexual behavior, five test-situations were used. Both males and females were divided in four experimental and one control group. All testing occurred in a rectangular mating arena (40–50–65 cm) that was not cleaned between mating sessions and therefore contained sex-related odor cues. In the control

<table>
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<td>The average numbers (± S.E.M.) of mounts (#M), intromissions (#IM) and ejaculations (#EJ), and the average duration in time (TIME), indicated in minutes (') and seconds (&quot;), of the different behavioral situations in males and females</td>
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<table>
<thead>
<tr>
<th>Males</th>
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<tr>
<td>#M</td>
<td>#IM</td>
<td>#EJ</td>
<td>TIME</td>
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<tr>
<td>Mount</td>
<td>19.3 ± 0.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8 IM</td>
<td>4.3 ± 0.9</td>
<td>8</td>
<td>0</td>
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<tr>
<td>1 EJ</td>
<td>3.7 ± 0.3</td>
<td>14.0 ± 1.5</td>
<td>1</td>
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<tr>
<td>2 EJ</td>
<td>10.3 ± 9.4</td>
<td>17.7 ± 3.8</td>
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Mount: mounts only; 8 IM: 8 intromissions; 1 EJ: one ejaculation; 2 EJ: two ejaculations.
group (males: \( n = 3 \), females: \( n = 3 \)), the animals were placed in the mating arena with clean bedding without presentation of a mating partner. In the four experimental groups, 5 min after the males were placed in the mating arena with clean bedding, the receptive female partner was introduced. The durations of the different tests and the numbers of mounts, intromissions and ejaculations, are presented in Table 1. The first experimental group consisted of males only allowed to mount \( (n = 3) \) and females \( (n = 3) \), which were only mounted, without intromissions. The males mounted estrous females with vaginal masks (tape covering the vagina and perineum) and the females were mounted by males with lidocaine-treated penises, unable to intromit. The second group consisted of males \( (n = 3) \) and females \( (n = 3) \) achieving and receiving eight intromissions, but no ejaculations. After eight intromissions, animals were removed from the arena. In the third group, males \( (n = 3) \) and females \( (n = 3) \) were tested until one ejaculation occurred. And finally in the fourth group, males \( (n = 3) \) and females \( (n = 3) \) were tested until two ejaculations had occurred.

After the test period, male and female rats were taken back to their home cages. Sixty min after replacing the animals to their home cages, they were anaesthetized using sodium pentobarbital (Narcoet, 30 mg/0.5 ml, i.p.), treated i.p. with 1 ml heparine (Organon Teknika, Boxtel, The Netherlands) to prevent excessive bloodclotting, and perfused transcardially with 0.1 M phosphate buffered saline (PBS), pH 7.3, followed by 400 ml 4% paraformaldehyde 0.1 M PBS, pH 7.5. Brains were removed and postfixed for 16–18 h at 4°C in the same fixative.

### 2.3. Additional female control groups

Since all females received hormone treatment with EB and P to induce sexual receptivity, additional control situations were included to study the effects of the hormone treatment on the distribution of Fos-IR. Three groups of females were tested. Two weeks after ovariectomy, all females received hormone treatment of EB and P, and mated with different male partners. One week later, in the first group, 4 h following progesterone administration, hormone-treated females \( (n = 2) \) were placed in the mating arena with clean bedding, for a period of 15 min, after which they were returned to their home cages. This procedure was similar to the procedure used for the group of control females described above. The second group consisted of hormone-treated females remaining in their home cages \( (n = 2) \). These females were sacrificed at the same time following the progesterone administration as the females that were placed in the mating arena, which added up to approximately 5 h and 15 min. A third group of females received no hormone treatment and remained in their home cages \( (n = 2) \). These females were sacrificed simultaneously with the females in the other two groups by transcardial perfusion as described above.

### 2.4. Immunocytochemistry

Coronal sections were cut at 75 µm using a vibratome (BioRad) and collected in 0.1 M PBS. Free-floating sections were washed twice in PBS and soaked for one h in incubation solution (PBS containing 0.1% bovine serum albumin and 0.5% triton X-100). Next, sections were incubated overnight on a shaker at room temperature with an anti-Fos antiserum raised in sheep (OA-11-824, Cambridge research Biochemicals, Northwich, UK), diluted 1:10,000 in incubation solution. Subsequently, sections were incubated in donkey anti-sheep (1:400 in incubation solution, Jackson) for 60 min at room temperature and in ABC-Elite (Vector 1:800 in PBS) for 120 min at room temperature. In between incubations, sections were washed in PBS. The peroxidase complex was visualized by exposure for 10 min to a chromogen solution containing 0.02% 3,3’-diaminobenzidine tetrahydrochloride (DAB) with 0.3% Nickel-ammonium sulfate in 0.05 M Tris-buffer (pH 7.6), followed by incubation for 10 min in chromogen solution with hydrogen peroxide (1:3000) to produce a blue-black product. The reaction was terminated by extensive washing in PBS. All sections were double-stained for calcitonin gene related peptide-immunoreactivity (rabbit polyclonal antiserum, Amersham, UK), of which the results will be described in detail in a separate report. Sections were then mounted on gelatin/chrom alum-coated glass slides, dried overnight, cleared in xylene, embedded with Entellan (Merek, Darmstadt, Germany) and coverslipped.

### 2.5. Analysis of Fos-IR in brain sections

For quantification of the numbers of Fos-IR neurons following the different behavioral situations, animals were given codes not related to their test-situations. Fos-IR were counted in the medial preoptic nucleus (MPN), posterodorsal medial amygdala (MEApd), posteromedial bed nucleus of the stria terminalis (BNSTpm), parvicellular subparafascicular nucleus (SPFp), and in the ventrolateral part of the ventromedial hypothalamic nucleus (VMHvl) in a standard area of 0.25 mm² (MPN, MEApd) or 0.125 mm² (BNSTpm, SPFp, VMHvl), in two adjacent sections representative for each brain area where differences in Fos-IR were observed, using a Zeiss light microscope and drawing tube. In the caudalventral part of the ventromedial hypothalamic nucleus (VMHcv) and the ventral premammillary nucleus (PMV), Fos-IR neurons were counted within the borders of the particular nucleus without using a standard area.

The data are presented as mean numbers (± S.E.M.) of Fos-positive cells in two adjacent sections. Comparisons between groups were statistically analysed using the Duncan multiple range test, with a 95% level of significance. Numbers of Fos-IR neurons in the additional control females are presented in Fig. 4 as means of Fos-positive cells in two adjacent sections. Since the groups each
3. Results

3.1. Numbers of Fos-IR neurons in males

Induction of Fos-IR was observed in the MPN, BNSTpd, MEApd, and SPFp following sexual behavior. Not all phases of sexual activity were followed by increased numbers of Fos-IR neurons, and this varied for the particular brain areas (Fig. 1A–D). In the male MPN (Fig. 1A), increasing numbers of Fos-IR neurons were observed following increasing sexual activity. The numbers of Fos-IR neurons were increased following mounts, but were further increased following intromissions or one or two ejaculations. In the BNSTpd (Fig. 1C), MEApd (Fig. 1E), and SPFp (Fig. 1G) a single or two ejaculations were followed by increases in the number of Fos-IR neurons. Although both one or two ejaculations resulted in increases in Fos-IR in all four brain areas, numbers of Fos-IR following two ejaculations were significantly higher in all regions compared to one ejaculation.

3.2. Numbers of Fos-IR neurons in females

In the female MPN (Fig. 1B), BNSTpm (Fig. 1D) and MEApd (Fig. 1F) the numbers of Fos-IR neurons were increased only following one or two ejaculations. In the SPFp (Fig. 1H), intromissions or successive numbers of ejaculation were followed by increases of Fos-IR cells. However, in none of the four brain regions were the numbers of Fos-IR neurons following two ejaculations higher than following one ejaculation.

3.3. Distribution of Fos-IR in other brain areas in males and females

Apart from these four areas, populations of Fos-IR neurons were observed consistently in other brain regions. In the male rat, Fos-IR neurons were situated in the posterodorsal preoptic nucleus (PD; Fig. 7F) and in the posterior nucleus of the amygdala (PA) only following ejaculation and not following mounting or intromissions.

In females, Fos-IR in the PA was also only detected following one or two ejaculations. However, in contrast to the males, Fos positive cells were detected in the PD in all females (Fig. 7A,C,E). In addition, in all female rats, induction of Fos-IR was observed in the ventral premamillary nucleus (PMV; Fig. 3H) and in the most caudalventral part of the VMH (VMHcv) close to the ventral border of the brain (Fig. 3D,E). This subregion is cytoarchitectonically different from the VMHvl and the PMV and can be observed as a dark-staining cell cluster in Giemsa-stained sections. In both the PMV and VMHcv, the numbers of Fos-positive cells in the control females did not differ from the number of Fos-IR cells in sexually-active females, and no differences were observed following the several types of sexual activity. In males, Fos-IR neurons were few in the PMV ($P < 0.001$; Fig. 3D) and VMHcv ($P < 0.001$; Fig. 3F).

In females, Fos-IR was observed in the ventrolateral region of the VMH (VMHvl; Fig. 3A), where a small but significant increase in the numbers of Fos-positive cells was detected in females following mounting (Fig. 2). Numbers of Fos-IR neurons were further increased following increasing sexual activity. Following intromissions, the number of Fos-IR cells was slightly higher, and a strong induction could be observed following two ejaculations. In addition to the increasing numbers of Fos-IR neurons, there was also a tendency towards stronger staining intensity of the Fos-IR cells with increasing sexual activity. No Fos-IR neurons were observed in the VMHvl in the male partners (Fig. 3C).
3.4. Fos-IR in the additional female control groups

Females that did not receive hormone treatment and remained in their home cages showed very few Fos-IR neurons in all brain areas. In contrast, in all females treated with EB and P, Fos-IR was induced in PD, VMHcv (Fig. 3D), and PMV (Fig. 3G), regardless whether the females were placed in a test-cage or remained in their home-cages.

Also in the MPN and BNSTpm, hormone treatment was followed by induction of Fos-IR (Fig. 4). Especially in the MPN, hormone treatment was followed by an increase in the number of Fos-IR neurons (Fig. 4). In addition, Fos-IR neurons were observed close to the ventricle in the periventricular region of the POA (Fig. 5C-E). In the MEApd, hormone treatment had no effect on Fos-IR. However, placement of the females in the test-cage caused an increase in Fos-IR compared to the Fos-IR in females that remained in their home cages (Fig. 4).

3.5. Clusters in the BNSTpm and MEApd

In several of the regions described above, the Fos positive cells were not evenly distributed over the particular brain area, but formed ‘clusters’ of Fos-IR cells in specific subregions. For instance, Fos- positive cells in females were not dispersed over the entire PMV, but appeared as a cluster of cells in the ventral part of the PMV (Fig. 3H). Although some males showed Fos-IR neurons in the PMV, the distribution never showed the same characteristic pattern as detected in females. Instead, Fos-IR cells were diffusely scattered over the entire PMV. Also in females, Fos-IR neurons were activated in a cluster in the VMHvl (Fig. 3B), and in a subregion referred to as the VMHcv (Fig. 3E). In the MPN of some of the female subjects, clustering of Fos positive neurons could be detected in similar regions in the brain of the male and female rat, with additional activated areas in females.

Specific clustering of Fos-IR cells was especially present in BNSTpm and MEApd. In the BNSTpm, two clusters of Fos-positive cells appeared in the male rat after ejaculation only. One of these clusters was situated dorsally in the rostral part of the BNSTpm, close to the ventricle (Fig. 6F). The other cluster was observed more ventrally in the caudal part of the BNSTpm, close to the fornix (Fig. 7F). The rostral cluster was also present in females following ejaculation. However, the caudal cluster was observed in all females regardless of the female following ejaculation, but showed no consistent appearance. In addition, following intromissions or mounts, Fos-IR neurons also appeared to be situated in this specific subregion although no clustering was apparent.

Although in the male rat mounting or intromissions were not observed to result in increased numbers of Fos-IR neurons, differences between the distributions of the neurons following mounts, intromissions, or ejaculations could be observed. In the MEApd of the male rat, mounting resulted in a homogeneous distribution of Fos-IR neurons (Fig. 8B), while following intromissions the Fos-positive cells tended to be located in the medial region close to the optic tract (Fig. 8D). After ejaculation, a cluster of Fos positive cells appeared in the lateral part of the MEApd (Fig. 8F). Following ejaculation by the male partner, a cluster of Fos positive cells, similar to the cluster observed in males following ejaculation, was present in females in the same lateral part of the MEApd (Fig. 8E). Also following mounts or intromissions, Fos-IR cells were mostly situated in the lateral part of this nucleus (Fig. 8A,C). Apart from some scattered Fos-IR cells, Fos-IR was not detected in the medial part of the MEApd.

4. Discussion

The present results confirm our previous findings that performance of mating including ejaculation dramatically increases Fos-IR in the MPN, BNSTpm, MEApd, and SPfp in male rats [6,7]. In addition, it was demonstrated that sexual stimulation increases the numbers of Fos-IR neurons in similar regions in the brain of the male and female rat, with additional activated areas in females. Differences between males and females were observed in the increases of Fos-IR following the different phases of mating performance.

We did not intend to make a quantitative comparison of the absolute numbers between males and females, since the differences in the gonadal steroid regimes of the intact males and the ovariectomised hormone-treated females make such a comparison virtually meaningless. Instead, a qualitative comparison between the sexes based upon the distribution of Fos-IR and the nature of the sexual activity inducing Fos positive cells in the analysed regions was
performed. Furthermore, possible sensory and hormonal stimuli in the different phases of male and female sexual behavior will be discussed in relation to the observed distribution of Fos-IR.

4.1. Fos-IR in females

Fos-IR in females following mating including ejaculation was induced in the MPN, BNSTpm, MEApd and SPFp, confirming observations by several investigators [10,12,33,34,38,46]. Although induction of Fos-IR in these specific brain areas was also observed in males, the successive elements of sexual behavior in females resulted in a different pattern of induction when compared with the induction pattern in males. In addition, increases in Fos-IR in females were detected in brain areas that were not activated in males, including the VMHvl, VMHcv and PMV. Apparently, the neural circuitry expressing Fos following sexual behavior in females contains a number of additional areas.

4.1.1. Hormonal stimulation

In the present study, Fos-IR was induced following hormone treatment with EB and P in the periventricular region of the MPN, BNSTpm, PD, VMHcv, and PMV. With the exception of the VMHcv, these brain areas are

Fig. 5. Photomicrographs illustrating the distribution of Fos-IR in two sections of the MPOA (150 μm apart). A,B: the distribution in OVX females that received no hormone treatment and remained in their home cages. C,D: the distribution following hormone treatment in OVX females that remained in their home cages. E,F: the distribution in OVX females that received hormone treatment and in addition were placed in the mating arena. aco = anterior commissure, och = optic chiasm, v3 = third ventricle. Scale bar = 300 μm.
The figures illustrate the distribution of ROS neurons in the caudal BST. In panels (A) and (C), arrows indicate the appearance of clusters of ROS neurons in this region. The arrows in panels (C) and (D) correspond to the immunohistochemical staining of ROS. The scale bar in (D) represents 40 μm.
Scale bar = 100 μm.

Figure 3. Photomicrograph illustrating the distribution of FO4-IR neurons in the MEAFL in laminae (left column) and medulla (right column). Arrows indicate regions of interest (C–D) or fixation (E–F).
known to contain large amounts of estrogen receptor containing neurons, and the distribution of the Fos-positive neurons strongly resembled the distribution of the estrogen receptor containing neurons [40]. Our findings were consistent with previous reports that treatment with EB activates Fos-IR in the hypothalamus and amygdala [16], or increases Fos mRNA levels in midbrain samples [4]. However, Gibbs et al. [13] reported contradictory findings, showing no increases in Fos mRNA levels or numbers of Fos-IR cells in hypothalamus or medial amygdala following treatment with EB or with EB and P. The contradiction may be a result from differences in the hormone treatments.

In the present study, a distinct cluster of Fos-positive cells was detected caudally from the VMH following hormone treatment. Based on its appearance in Giemsa-stained sections, we identified this area as the most caudal part of the ventral division of the VMH, consisting of densely-packed, darkly-staining cells. In the cytoarchitectonic atlas of the hypothalamus of Bleier et al. [3], this subarea is included in the ventromedial nucleus (Plates 21 and 22). In our opinion the VMHcv is not only cytoarchitectonically different from the VMHvl and PMV, but also the population of Fos-IR cells in this area is separate from the populations in the VMHvl and PMV. Further research will be necessary to study the specific anatomical afferent and efferent relationships of this particular region, as well as the occurrence of estrogen and androgen receptor containing neurons. Preliminary data revealed the presence of calcitonin gene related peptide-containing cells in the female VMHcv, and colocalization of calcitonin gene related peptide and Fos following hormone treatment or sexual activity (pers. obs.).

4.1.2. Lordosis behavior

The VMH has been identified as a major site for regulation of lordosis behavior. Lesions of the VMH dramatically reduce lordosis behavior [31], while electrical stimulation facilitates the expression of lordosis in hormone-primed females [32]. Furthermore, lordosis can be potentiated by central application of several neuropeptides and neurotransmitters in the VMH [19]. In the present study, Fos-IR in the female VMHvl was induced following lordosis behavior in females which were mounted by males. This suggests that the induction of Fos-IR in this area is correlated with the expression of lordosis behavior. Stronger induction of Fos-IR was detected following intromissions, although these were not accompanied by a higher expression of lordosis behavior. These findings are consistent with observations by Pfau et al. [33] and by Rowe and Erskine [38], that manual flank stimulation or mounting by males induced lower numbers of Fos-IR in this region compared to the number of Fos-IR neurons following vaginocervical stimulation by manual probing or by intromissions including ejaculation.

The significance of the Fos-IR observed in the VMHvl can be interpreted in different ways. Fos-IR is possibly a reflection of afferent sensory stimulation, consisting of somatosensory stimulation by the mounting males and by vaginocervical stimulation from intromissions. However, Rowe and Erskine [38] could reveal no effect of disturbance of the relay of afferent sensory input resulting from vaginocervical stimulation by transection of the female pelvic nerve on the Fos-IR in the VMHvl of mated females. In contrast, Wersinger et al. [46] did report a reduction of Fos-IR as an effect of the transection of the pelvic nerve. Yet, the numbers of Fos-IR neurons in the VMHvl were still higher in mated transected females, compared to unpaired control females. Thus, induction of Fos-IR in the VMHvl does not seem to be solely a reflection of vaginocervical sensory stimulation. In addition, it seems unlikely that the Fos-IR is simply a reflection of motor activity related to lordosis behavior. In the present study, eight intromissions were followed by a stronger induction of Fos-IR than mounts only, while intromissions were not accompanied by a higher expression of lordosis behavior.

Furthermore, the induction of Fos-IR in the VMHvl may be a reflection of the convergence of sensory and hormonal cues possibly related to the onset of lordosis behavior. In favor of this assumption, increases in the numbers of Fos-IR neurons in the VMHvl and a tendency towards a stronger staining intensity of Fos-IR cells was observed with increasing or stronger sexual activity of the females. The stronger staining intensity may reflect a stronger activation of the Fos-IR neurons [20], which possibly reflects some aspects of the internal motivational state of the female concerning the display of lordosis behavior. In support of this suggestion, Rajendren et al. [36] reported that repetitive mating enhances lordosis behavior, suggesting an increase in motivation of the female to perform lordosis behavior. Positive feedback mechanisms may be active in these situations, similar to what has been observed in feeding behavior [47]. In addition, Rajendren et al. [36] showed that lesions of the VMH completely abolish this effect of repetitive mating.

4.1.3. Vomeronasal stimulation

The experimental design of the test-situation in which the females were only mounted without intromissions involved females that were mounted by males with lidocaine treated penises. These females extensively displayed chemosensory investigation, especially since the males were somewhat inactive probably as a result of the lidocaine treatment. In these females, that were only mounted, Fos-IR was slightly induced in the BNSTpm. The increase in Fos-IR in the female BNSTpm may therefore reflect the display of chemosensory investigation. Females receiving eight intromissions displayed only low levels of chemosensory investigation due to the short time-period of testing and the high sexual activity of the males. Accordingly, no
increased Fos-IR was detected in the BNSTp in those females.

In addition, no increased number of Fos-IR neurons was observed in the MEApd in females that were mounted, which indicates that the MEApd is not strongly affected by chemosensory investigation in females. This finding is supported by other studies, since Tetel et al. [42] reported no increase of Fos-IR in the MEApd following exposure of females to anesthetized males. Furthermore, Rajendren and Moss [35] showed that removal of the vomeronasal organ did not significantly reduce Fos-IR in the MEApd following mating in female rats.

In the present study, placement of the females in the mating arena resulted in increased numbers of Fos-IR neurons in the MEApd, compared to females that remained in the home cage. Apparently, Fos-IR was induced by the presentation of the familiar mating arena. Additional induction of Fos-IR by presentation of a male did not occur.

4.1.4. Vaginocervical stimulation

Although other researchers [10,33,34,38,41,46] observed Fos-IR in the MPOA, BNST and MEA following vaginocervical stimulation, we did not observe any induction of Fos-IR in the MPN, MEApd or BNSTp following eight intromissions. This contrast can be explained by the differences in the amount or intensity of vaginocervical stimulation used to induce Fos-IR in the different studies. The amount of vaginocervical stimulation by eight intromissions in our study is low compared to the stimulation used in other studies, which consisted of high numbers of manual stimulations (30 [42,43] to 50 [33]), high numbers of intromissions (30-36 [42]), or by intromissions including ejaculations [10,38].

In the present study, an increase in numbers of Fos-IR neurons was observed following ejaculation in the MPN, BNSTp and MEApd, even though it was only preceded by an average of 14 intromissions. Considering the observed increase of Fos-IR following ejaculation, but not following eight intromissions, it appears that the vaginocervical stimulation caused by the ejaculation of the male is a much stronger genitosensory stimulation than the stimulation by a limited number of intromissions without ejaculation. It has been reported that extended uterine contractions may occur in the female during mating [44], which may result in afferent sensory information that distinguishes ejaculation from a series of intromissions.

Sensory input arising from vaginocervical stimulation is mainly relayed via the pelvic nerve [17,30]. These fibers can be described as afferent parasympathetic directed at the L6-S1 segments of the spinal cord [45]. The involvement of the pelvic nerve in the induction of Fos-IR in the MPOA, BNST and MEA in the female is supported by the reduction of Fos-IR following transection of this nerve [38,46]. Afferent genitosensory input that is relayed through the pelvic nerve may ascend via the spinal cord [5,26] to influence directly the caudal diencephalon and especially the SPFP [22], or indirectly via the solitary nucleus [15,29]. In the present study, numbers of Fos-IR neurons were increased in the SPFP of females that received intromissions or several successive ejaculations. Therefore, Fos-IR in the SPFP probably reflects sensory input as a consequence of vaginocervical stimulation relayed via the pelvic nerve.

4.2. Fos-IR in males

4.2.1. Male copulatory behavior

The results in the present study demonstrate that all behavioral elements of the consummatory phase contributed to the activation of Fos-IR in the male MPN, including mounting behavior without intromissions. Increasing sexual activity reflected by increasing numbers of mounts, intromissions and ejaculations resulted in a clear increase in the number of Fos-IR neurons.

The complex anatomical connections of the MPN [39] and the presence of dense populations of androgen and estrogen receptor-containing neurons [1,40], suggest the MPN to be in an ideal position to integrate vomeronasal sensory, genital sensory and hormonal stimuli necessary for the control of male copulatory behavior. The convergence of the sensory and hormonal stimuli may be reflected by induction of Fos-IR in the MPN. In a previous report [6,7], we demonstrated that Fos-IR was not increased in the MPN following vomeronasal stimulation. In addition, Baum and Everitt [2] demonstrated that lesions in the MEA or in the central tegmental field were not sufficient to reduce Fos-IR in the MPN following mating. Therefore, it seems unlikely that the Fos-IR in the MPN reflects solely afferent inputs. We suggest that Fos-IR probably reflects some kind of motivational state induced by converging afferent sensory and hormonal inputs that results in the integration of motor responses involved in the consummatory phase of male copulatory behavior and the facilitation of genital reflexes.

4.2.2. Vomeronasal stimulation

In the present study, the main focus was on the induction of Fos-IR caused by consummatory aspects, and there were no specific test-situations included to study the effects of vomeronasal inputs on the induction of Fos-IR. The amount of vomeronasal stimulation was minimal in the experimental condition where males were allowed to display only mounting behavior without intromissions when the female's vaginas were taped. Also during the very short time that eight intromissions were performed (4 min), vomeronasal stimulation was low. Accordingly no increases in numbers of Fos-IR were observed in the BNSTp or MEApd. The distribution of Fos-IR neurons in the MEApd resembled the distribution that was previously observed following chemosensory investigation [6]. It is therefore possible that the distribution of Fos-IR neurons following intromissions in the MEApd is a result of vomeronasal stimulation.
4.2.3. Ejaculation-related clusters of Fos-IR neurons

Increasing numbers of ejaculations caused increases of Fos-IR in distinct subareas of the male rat brain. Fos-IR was strongly increased in the SPFp, the PD, the PA, and in subareas of the MEApd and the BNSTpm.

In the MEApd, intromissions without ejaculation resulted in induction of Fos-IR in the medial part of the MEApd, very close to the optic tract. In addition, high numbers of Fos-IR neurons were observed in the MEApd following ejaculation. These ejaculation-related Fos-IR neurons were situated in the lateral part of the MEApd, separately from the Fos-IR neurons observed following intromissions. This characteristic pattern has also been described by other investigators in rats [2] and is similar to the pattern observed in male hamsters, where two clusters of cells appear following mating including ejaculation in the lateral part of the MEApd [11,18,48].

In the male BNSTpm, two clusters of Fos-IR related to ejaculation were observed. One cluster was situated in the rostral extension of the BNSTpm close to the ventricle. This cluster was also observed in male hamsters following mating including ejaculations [11]. In male rats we observed an additional cluster of Fos positive cells in the caudal aspect of the BNSTpm, in a subregion situated close to the fornix, following ejaculation.

The present study demonstrates that especially the MEApd, but also the BNSTpm, consist of several subregions which are involved in different aspects of sexual activation since specific clusters of Fos-IR neurons appear only following ejaculation. Furthermore, we assume that ejaculation differs from intromissions in terms of afferent sensory stimulation, since intromissions and ejaculation result in a different pattern of activated neurons in different brain areas.

Sensory stimulation of the penis, as occurring during intromissions, results in afferent somatosensory stimulation relayed through the pudendal nerve [8,9,23], ascending from the lumbar and sacral parts of the spinal cord [41] directly or indirectly to the SPFp [22,27]. A different type of activation may occur by afferent viscero-sensory stimulation related to ejaculation from the internal reproductive organs. The latter may be relayed via the pelvic nerve [8,9], and may ascend from the caudal lumbar and sacral (L6-S1) segments of the spinal cord [24,26,28,41] also to the SPFp [22,27]. This ascending information may reach the SPFp directly from the spinal cord [5,22]. Both kinds of ascending sensory information may influence similar parts of the brain. Yet the appearance of specific clusters of Fos-IR in MEApd, BNSTpm, and SPFp can be explained only by assuming that genital somatosensory and viscero-sensory messages ascend along parallel pathways in such a way that specific clusters of Fos-IR neurons appear as a result of the visceral input, parallel to or within the larger areas that are affected by somatosensory afferent input.

This assumption is further supported by the observation that Fos-IR in females, presumably also reflecting afferent viscero-sensory input via the pelvic nerve, is induced in the same subregions of the MEApd, BNSTpm, and SPFp. The only exception is the caudal cluster of Fos-IR neurons in the BNSTpm which is less evidently present in females. This may be a result of the sexual dimorphic character of the BNSTpm. Especially since relatively thick (75 μm) sections were studied, we might have failed to detect the small cluster in the female caudal BNSTpm. However, a similar cluster of activated neurons in this part of the BNSTpm was reported by Polston and Erskine [34] using egr-1 as a marker for neural activation.

Besides differences in afferent sensory inputs, intromissions and ejaculations are different in terms of motor output. Ejaculation-related Fos-IR is unlikely to reflect the motor output following ejaculation, since Fos-IR was induced in the same subregions in females showing an entirely different motor pattern.

5. Conclusions

In conclusion, successive elements of copulatory behavior were followed by increases in Fos-IR in generally the same brain areas in male and female rats. The BNSTpm appears to be involved in chemosensory investigation, while specific distinct subregions are only activated following ejaculation. In addition, the SPFp and the lateral part of the MEApd appear to be involved in the integration of viscero-sensory input. The neural circuits underlying sexual behavior in males and females appear to be similar in terms of integration of sensory information. In males, however, the MPN may be regarded as an important brain region for the integration of sensory and hormonal stimulation leading to the onset of male sexual behavior. While in females the VMHvl appears to be involved in the integration of stimuli leading to the onset of lordosis behavior.

In addition, following successive elements of sexual behavior, Fos-IR was induced in different subregions within larger brain areas involved in the regulation of sexual behavior. This was observed in the BNSTpm and MEApd, as well as in the VMH and PMV.

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