DISTRIBUTION OF FOS IMMUNOREACTIVITY FOLLOWING MATING VERSUS ANOGENITAL INVESTIGATION IN THE MALE RAT BRAIN

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Abstract—In the present study a detailed quantitative analysis was made using Fos as a marker for neural activation to define which subregions in the neural circuitry underlying male sexual behavior are involved in display of anogenital investigation versus copulation. Neural activity was differentially distributed following anogenital investigation versus mating and was restricted to specific subdivisions that form a heavily interconnected network. Chemosensory investigation increased neural activity in the posteromedial subdivision of the bed nucleus of the stria terminalis and the posterodorsal subdivision of the medial amygdala, brain regions that receive chemosensory signals processed through the olfactory bulbs, presumably reflecting the acquisition of chemosensory signals or the display of anogenital investigation. However, other sensory signals or sexual experience may also have contributed to the induction of neural activation in these brain areas. Moreover, consummatory behavior increased neural activity in the subparafascicular nucleus, a brain region that receives genital sensory inputs. In turn, this brain region projects to the medial preoptic nucleus and posterior nucleus of the amygdala, where neural activity was also abundant only following copulation. In addition, clusters of neurons were activated in the posteromedial subdivision of the bed nucleus of the stria terminalis and posterodorsal subdivision of the medial amygdala following consummatory behavior.

The present study provides an anatomically detailed picture about the distribution of neural activation following sexual behavior in the rat, specifically in relation to differences following anogenital investigation versus mating. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: medial preoptic area, medial amygdala, bed nucleus of the stria terminalis, midbrain, chemosensory signals, genitosensory signals.

Knowledge of the neural regulation of male sexual behavior in the rat is largely based on studying copulatory behavior following electrolytic or chemical lesions, electrical stimulation or implantation of small quantities of steroid hormones in specific brain areas (reviewed in Sachs and Meisel4*). However, in recent years, several investigators have used Fos immunoreactivity (IR) to map functional neural circuits underlying copulation in the male rodent brain. Immunocytochemical visualization of Fos, the protein product of the immediate early gene c-fos, has been widely used as a marker for the activation of neurons.22,36 Copulation increased Fos-IR in the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BNST), medial amygdala (MEA) and central tegmental field (CTF) of male rats.1,41,49,50,22,28,29,36 Hamsters,16,28,29,50 and gerbils.19,16,28,29,36 These brain regions act in concert to regulate male sexual behavior.43 However, male sexual behavior is not just one behavior. Instead it is a complex of different behaviors, including anogenital investigations such as chemosensory investigation of the female, and consummatory behaviors, i.e. mounting, intromissions and ejaculations.15,43 Previous studies in male rat,1 hamster1,6,28 and gerbil19 using Fos-IR as a marker for neural activation have indicated that various elements of sexual behavior result in different numbers of activated neurons. The present study provides an anatomically detailed picture about differences in neural activation in the male rat by determining differences in patterns of neural activation, in addition to differences in numbers of activated neurons, in subregions of the MPOA, BNST, MEA and CTF following appetitive versus consummatory aspects of sexual behavior. The approach was to compare Fos expression in males displaying different aspects of sexual behavior. To determine Fos-IR following
consummatory behavior, males were allowed to mate with a receptive female. Additional males were exposed to a non-receptive female to identify Fos-IR induced by anogenital investigation alone. To test for effects on neural activation of prior mating experience and conditioned sexual responses, Fos-IR in sexual experienced and naive males was compared.

Table 1. Schematic presentation of sexually relevant stimuli and behaviors expressed in the five groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mating (mounts, intromissions, ejaculations; estrous female)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anogenital investigation (anestrous female)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Odor-rich environment</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Mating experience</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Handling and exploration</td>
<td>-</td>
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Groups A–C, n=5; Groups D and E, n=4.

Experimental procedures

Animals

Male Wistar rats (n=23, 300 g, three months old), obtained from the local breeding facilities of the University of Nijmegen, were group-housed separately from females in an artificially lit room on a reversed 12:12-h light/dark cycle. Food and water were available at all times. Male Wistar rats (200 g) were bilaterally ovarioctomized and used as stimulus animals. Sexual receptivity was induced by administration of 50 µg estradiol benzoate in 0.1 ml arachidis oil 48 h prior to testing, followed by 500 µg progesterone in 0.1 ml arachidis oil 4 h before testing. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Experimental protocols

Five different test situations were used (see Table 1). All testing occurred 5–6 h after lights off, and the same procedure was used for all situations. Briefly, males were placed in the testing environment for 5 min, after which an estrous, anestrous or no female was presented for 30 min. Males were returned to their home cages and perfused 60 min after the end of the test.

Groups A-C consisted of sexually experienced males. Animals received mating experience during four pre-test mating sessions. Males were considered sexually experienced when displaying multiple ejaculations and the first ejaculation within 10 min after onset of the last two mating tests. Males were tested in the same environment in which they gained mating experience. This rectangular mating arena (40 x 50 x 65 cm2) was not cleaned between mating sessions to create a test environment rich with sex-related odors. Males in Group A (n=5) mated with a receptive estrous female, displaying both anogenital investigation and copulation including ejaculations (3.4 ±0.2). In Group C (n=5), males were placed alone in the familiar mating arena without a female to study Fos-IR induced by the odor-rich mating arena and by handling. In Group B (n=5), sexually naive males interacted with a non-receptive anestrous female. These males displayed only chemosensory investigation (taping the vaginal region of the female, placing a female into the male's genital region and display chemosensory investigation equivalent to males paired with estrous females.12

In Groups D and E, sexually inexperienced rats were placed in a clean test cage similar to the mating arena during four pre-test exposures and the final test. This test cage was never inhabited by estrous females and was free of sex-related odors. As in Groups A-C, bedding was not changed between tests. Sexually inexperienced males in Group D (n=4) interacted with a non-receptive anestrous female during the final test, similar to sexually experienced males in Group B, and also during four pre-test sessions. These males displayed anogenital investigation, but no mounting behavior or penile intromissions. Sexually inexperienced males in Group E (n=4) were placed alone in the familiar test cage without presentation of a female to serve as controls.

One hour after the end of the test, males were anesthetized using sodium pentobarbital (30 mg/0.5 ml, i.p.), treated with heparine (1 ml, i.p.; Organon Teknika) to prevent excessive blood clotting and perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.3), followed by 400 ml Zamboni fixative (1.8% paraformaldehyde with 7.5% picric acid in PBS, pH 7.5). Brains were removed and postfixed for 16–18 h at 4°C in the perfusion fixative.

Immunocytochemistry

Coronal sections were cut at 75 µm using a Vibratome (Lancer series 10) and collected in PBS. Every second section was Nissl stained using Giemsa solution. The remaining sections were washed in PBS and soaked for 1 h in incubation solution (PBS containing 0.1% bovine serum albumin and 0.5% Triton X-100), with 5% normal horse serum. Subsequently, sections were incubated overnight on a shaker at room temperature with an anti-Fos antiserum raised in sheep (1:2000 in incubation solution; Cambridge Research Biochemicals, OA-11-823, batch: 07583/2351). Next, sections were incubated in horse anti-sheep immunoglobulin G (1:100 in incubation solution; Radboud H01-140987) and in sheep peroxidase-antiperoxidase (1:600 in incubation solution). Subsequently, sections were washed extensively in PBS. The peroxidase-antiperoxidase complex was visualized by exposure for 10 min to a chromogen solution consisting of 0.02% 3,3'-diaminobenzidine tetrahydrochloride with 0.3% nickel ammonium sulfate in 0.05 M Tris buffer (pH 7.6), followed by incubation for 10 min in chromogen solution containing hydrogen peroxide (0.03%) to produce a blue–black reaction product over Fos-containing nuclei. The reaction was terminated by extensive washing in PBS. Following staining, sections were mounted on gelatin/chrome–alum-coated glass slides, dried overnight, cleared in xylene, embedded with Entellan (Merck) and coverslipped.

Initially, one of the goals of the present study was to investigate the neural activation of luteinizing hormone-releasing hormone (LH-RH)-containing neurons, since copulation or exposure to a female induces release of luteinizing hormone.6,18,26,51 Therefore, all sections were
double-stained for LHRH (1:1000 in incubation solution; Eurodiagnostics, PLR lot 04) using the same protocol described above with unenhanced 3,3'-diaminobenzidine, to determine co-localization of LHRH and Fos-IR. LHRH-containing neurons in the medial septum, nucleus of the diagonal band, MPOA and anteroventral preoptic nucleus were examined for Fos-IR after mating. No double-labeled LHRH-containing neurons were detected following any of the behavioral tests, in agreement with reports in male hamsters\textsuperscript{11,12} and ferrets.\textsuperscript{2} However, LHRH-IR fibers traverse the populations of Fos-IR neurons in the postero medial BNST (BNSTpm) and posterodorsal MEA (MEApd) following consummatory or anogenital investigation.

**Analysis of Fos immunoreactivity in brain sections**

The number of Fos-IR neurons was counted in selected subnuclei of the amygdala, preoptic area, BNST and CTF. In the amygdala, numbers of Fos-positive neurons were counted in the bed nucleus of the accessory olfactory tract, posterior nucleus of the amygdala (PA), postero medial cortical nucleus and in four subdivisions of the MEA: anterodorsal, anteroventral, posterodorsal (MEApd) and posteroven tral. In the BNST, Fos-IR neurons were counted in the anteromedial (BNSTam), anterolateral (BNSTal), posteromedial (BNSTpm) and posterolateral (BNSTpl) subdivisions. In addition, Fos-IR neurons were counted in the medial preoptic nucleus (MPN), a subdivision of the preoptic area, and in a subdivision of the CTF. In the CTF, Fos-IR neurons were localized in a small horizontal layer dorsal to the medial lenticular nucleus (ml), extending rostro medi ally and caudo laterally between the fasiculus retroflexus and the peripeduncular nucleus. This population of Fos-IR neurons largely corresponds to the parvicellular part of the subparafascicular thalamic nucleus (SPFp) and is referred to herein by that name. However, Fos-IR also extends further caudally ventral to the ml within the boundaries of the zona incerta, as well as caudo laterally within the boundaries of the peripeduncular nucleus.

Subdivisions of the MEA and BNST were identified from adjacent Giemsa-stained sections and were based on studies by Swanson,\textsuperscript{47} Paxinos and Watson,\textsuperscript{39} Krettek and Price,\textsuperscript{31,36} De Olmos et al.,\textsuperscript{38} Ju and Swanson,\textsuperscript{24} and Mogas et al.\textsuperscript{29} The nomenclature used for subdivisions of the MEA was largely based on the works of Swanson\textsuperscript{47} and De Olmos et al.\textsuperscript{38} In the BNST, a simplified nomenclature was used. The BNSTal corresponds to the anterolateral area described by Ju and Swanson,\textsuperscript{24} and to part of the lateral division defined by Krettek and Price.\textsuperscript{31,36} The BNSTpm corresponds to the antero dorsal area described by Ju and Swanson.\textsuperscript{24} The BNSTpl consists of Ju and Swanson's interfascicular nucleus and the transverse nucleus.\textsuperscript{24} The BNSTpm corresponds to the principal (encapsulated) nucleus of the posterior division from Ju and Swanson.\textsuperscript{24}

Fos-IR neurons were counted unilaterally in two adjacent sections from each brain region in each rat, using a Zeiss light microscope and drawing tube. The borders of the selected brain areas were drawn from the alternating Giemsa-stained sections. Subsequently, total surface areas (in mm$^2$) of the quantified brain regions were calculated for each section with the aid of Kontron Videoplan equipment. Results from two counts in each animal were averaged and data are presented as mean $\pm$ S.E.M. Fos-IR cells per mm$^2$ in each group. Due to large variance in some experimental groups the data were not normally distributed, and were statistically analysed using the non-parametric Welch test. Post hoc comparisons were based on the Hochberg method using the 0.05 level of significance.\textsuperscript{21} All groups were compared to sexually inexperienced controls (Group E). Group A was compared with Group B to evaluate neural activation following consummatory versus anogenital investigation, and Group B was compared with Group D to test for effects of experience and odor-rich environment.

In addition, Groups B and C were compared in the BNSTpm and MEApd using the Mann–Whitney test with a 0.05 level of significance.

**Abbreviations used in the figures**

- aco: anterior commissure
- cc: corpus callosum
- CEA: central nucleus of the amygdala
- cpd: cerebral peduncle
- f: fasciculus retroflexus
- fx: fornix
- IA: intercalated nuclei of the amygdala
- Lsv: ventral part of the lateral septum
- och: optic chiasm
- opt: optic tract
- PAG: periaqueductal gray
- PD: posterodorsal preoptic nucleus
- PMV: ventral premammillary nucleus
- SCN: suprachiasmatic nucleus
- sm: striatum
- SNe: substantia nigra, compact part
- SNr: substantia nigra, reticular part
- st: striatum
- STN: subthalamic nucleus
- v3: third ventricle
- vi: lateral ventricle

**RESULTS**

**Amygdaloid complex**

In the bed nucleus of the accessory olfactory tract, postero medial cortical nucleus, anterodorsal, anteroventral and posteroven tral subdivisions of the MEA, there were no differences in Fos expression between groups (Fig. 1). However, mating enhanced Fos expression in the PA and MEApd. In the PA (Fig. 1D), the number of Fos-IR neurons increased significantly in males mated to ejaculation (Group A). Likewise, in the MEApd (Fig. 1C), copulation significantly increased Fos-IR (Group A). Moreover, anogenital investigation in sexually experienced males exposed to an anestrous female (Group B) increased Fos above inexperienced controls (Group E), but below that of copulating males (Group A). However, anogenital investigation in experienced males failed to increase Fos expression above that in experienced males that were placed alone in the odor-rich test environment (Group C). Effects of prior sexual experience or odor-rich environment were not detected between Groups B and D, or C vs. E.

In addition to differences in the number of Fos-IR neurons in experienced males following mating and anogenital investigation, differences in the distribution of Fos-positive neurons were observed in the MEApd. Following consummatory behavior (Group A), a cluster of Fos-IR neurons was present in the lateral zone of the MEApd (Figs 4K, 5D). This cluster appears to correspond to a group of dark-stained cells as observed in Giemsa-stained sections (Fig. 4L). In contrast, following anogenital investigation (Groups B and D), Fos-positive neurons in the MEApd were restricted medially, close to the optic tract (Figs 4J, 5C). In all other males (Groups C and
Fos-IR neurons were scattered throughout the nucleus.

Bntrix nucleus of the stria terminalis

Mating failed to increase Fos-IR in the BNSTal (Fig. 2A). In the BNSTam (Fig. 2A) and BNSTpl (Fig. 2B), Fos-IR increased only following copulation (Group A). However, in the BNSTpm, Fos-IR was observed following both copulation (Group A) and anogenital investigation in sexually inexperienced males (Group D). In sexually experienced males, Fos induction following anogenital investigation (Group B) failed to reach a level significantly different from that in males mating to ejaculation (Group A), or from that in inexperienced males following anogenital investigation (Group D). There were also no differences in Fos expression between naive and experienced males that were not presented with a female (Groups C vs E).

In addition, differences in the distribution of Fos-positive cells were observed in the BNSTpm. Following copulation, two clusters of Fos-IR neurons were present. One was situated in the rostral subdivision of the BNSTpm, close to the lateral ventricle (Fig. 4E), while the other cluster was located in the caudal subdivision of the BNSTpm, close to the fornix (Figs 4H, 5B). Following anogenital investigation, Fos-positive neurons were evenly distributed throughout the BNSTpm and no discernible clusters of Fos-IR neurons were observed (Figs 4D, G, 5A).
Fos-IR following appetitive vs consummatory behavior

Fig. 2. Mean numbers of Fos-IR neurons per mm² (± S.E.M.) in specific subregions of the rostral (A) and caudal (B) BNST, with camera lucida drawings of adjacent Giemsa-stained sections (A–D), illustrating the subregions where the Fos-IR neurons were counted. Scale bar=1.0 mm. *P<0.05, **P<0.001.

Fig. 3. Mean numbers of Fos-IR neurons per mm² (± S.E.M.) in the MPN (A) and SPFp (B), with camera lucida drawings of adjacent Giemsa-stained sections, illustrating the subregions where the Fos-IR neurons were counted. Scale bar=1.0 mm. **P<0.001.

Prefrontal area

In the MPN, increased Fos-IR was detected only following consummatory behavior (Group A; Figs 3A, 4B). No regional differences in the distribution of Fos-positive neurons were observed between the central, medial and lateral parts of the MPN as defined by Simerly and Swanson.40 However, a cluster of Fos-IR neurons was observed in the posterodorsal preoptic nucleus (PD) following consummatory behavior (Group A; Fig. 4H).

Subparafascicular thalamic nucleus

In the SPFp, Fos-IR increased only in males mating to ejaculation (Group A; Fig. 3B). These Fos-positive neurons were distributed in a small horizontal layer dorsal to the ml (Figs 4N, 5).

DISCUSSION

The present study demonstrates neural activation in the MPOA, BNST, MEA and CTF, in agreement with previous reports on mating-induced Fos-IR in male rats, hamsters and gerbils.14,16,19,28,29,41,49,50 These data, in turn, have relevance to earlier reports on the disruption of sexual behavior following lesions and the facilitatory effects of electrical stimulation.43 Moreover, neural activity was restricted within these brain regions to specific subdivisions that form a heavily interconnected network. In addition, the subdivisions were differentially activated following
Fig. 4. A series of photomicrographs illustrating the distribution of Fos-IR neurons in the MPN (A, B), rostral BNSTpm (D, E), caudal BNSTpm (G, H), MEApd (J, K) and SPFp (M, N). The left column shows the distribution in experienced males that interacted with anestrous females (Group B), while the middle column illustrates the distribution in mating males (Group A), in representative animals. Photomicrographs of Giemsa-stained sections are included (right column, C, F, I, L, O), illustrating the architecture of each subnucleus. Scale bar=500 μm.
compensation
differences in learning
excitatory

In contrast, compensation and facilitation are ac-

mammalian brain, the distribution of neuronal activity
during aversive conditioning is more robust in the
task-compensation than in the control group.

However, the present study provides an
intrinsic neural activity. In the control group, more
neurons were activated in the BST than in the NAc
during the aversive conditioning, suggesting that
the BST is involved in the expression of aversive
conditioning.

The results show that the BST is
involved in the expression of aversive
conditioning. Furthermore, the BST
appears to be a critical region for
aversive conditioning, as it is
activated during the expression of
aversive conditioning.

In conclusion, the present study
provides evidence for the role of the
BST in the expression of aversive
conditioning.
Anogenital investigation

In the present study, anogenital investigation resulted in a pattern of activated neurons that was distinctly different from the distribution following consummatory behavior. Chemosensory investigation induced neural activation in the MEApd and BNSTpm, interconnected brain regions that receive direct and indirect olfactory inputs from the AOB. The distribution of activated neurons in the medial part of the MEApd close to the optic tract corresponds to the distribution of afferent fibers relaying vomeronasal input from the AOB. Similar findings have been reported in male hamsters following exposure to female hamster vaginal fluid. Likewise, removal of the vomeronasal organ decreased neural activation in the MEA induced by female hamster vaginal fluid. In rats, Baum and Everitt demonstrated decreased Fos-IR in the MEA following unilateral olfactory peduncle lesions. Collectively, these results using Fos to map functional neural circuits suggest that Fos expression following anogenital investigation reflects acquisition of chemosensory signals. However, additional sensory signals and sexual experience also appear to contribute to the induction of Fos following anogenital investigation. In experienced males, neural activation in the MEApd following anogenital investigation was different from that following exposure to the odor-rich environment in which the males gained sexual experience. Moreover, anogenital investigation did not increase neural activity in the MEA and BNST in males placed in a clean test cage. In contrast, a recent report by Baessler and Baum demonstrated neural activation in the MEA and BNST in males placed in soiled bedding from anestrous or estrous females. A possible explanation for these contrasting findings is the strength of the odor cues. Bedding used in the present study was not collected from estrous females. Therefore, the display of anogenital investigation and the resulting Fos-IR in males with an estrous female, bearing in mind that there might be a qualitative difference in actual chemosensory input.

Consummatory behavior

Neural activation following consummatory behavior seems to be consistent with the acquisition of genital and somatosensory signals. Consummatory behavior is followed by a different distribution of neural activation compared to Fos-IR related to olfactory signals associated with anogenital investigation. The present study demonstrated Fos-IR following copulation, but not following anogenital investigation, in the MEApd and BNSTpm. Fos-IR in males with an anestrous female can be compared to males with an estrous female, bearing in mind that there might be a qualitative difference in actual chemosensory input.
In the MEApd and BNSTpm, Fos-positive neurons were differently distributed following mating versus anogenital investigation. Clusters of Fos-positive neurons were noted in the MEApd and BNSTpm only following copulation. A similar distribution of Fos-positive neurons in the MEApd and BNSTpm has been observed in male hamsters\textsuperscript{16,28} and gerbils.\textsuperscript{19} In the MEApd, a cluster of Fos-positive cells was present in the lateral part of the MEApd following copulation, whereas anogenital investigation induced Fos mediolaterally within this nucleus. This suggests that neural activation in the lateral part of the MEApd is related to genital sensory inputs. Therefore, the MEApd can be further divided into a medial odor-sensitive area and a lateral genitosexual zone. In female rats, a similar distribution of activated neurons in the lateral part of the MEApd has been reported.\textsuperscript{13,40} and transection of the pelvic nerve reduced Fos-IR in the MEApd.\textsuperscript{42,49} In females, like in males, the pelvic nerve relays visceral sensory input from the internal reproductive organs.\textsuperscript{7,14,23} In a similar manner, we hypothesize that neural activation in the lateral part of the MEApd in male rats is related to visceral sensory stimulation relayed via the pelvic nerve.

Likewise, the BNSTpm contained two clusters of Fos-IR cells following consummatory behavior, suggesting that these clusters are activated by genital stimulation. At present, there is no anatomical evidence for direct projections relaying genital sensory inputs to the BNSTpm. However, the BNSTpm is heavily interconnected with the MEApd and PA,\textsuperscript{3,4} both of which receive genital sensory inputs from the SPFp.\textsuperscript{3,4,48,52}

The MPN is a brain region that receives both olfactory and genital sensory signals. It has reciprocal connections with the BNSTpm, MEApd, PA and SPFp.\textsuperscript{5,45,46} Moreover, the MPN is in a well-placed position to regulate sexual behavior regarding its connections with regions in the brainstem and spinal cord that are involved in the motor aspects of copulation. In the present study, neural activation was not induced in the MPN following chemosensory investigation, in agreement with previous reports,\textsuperscript{1} but was only detected following consummatory behavior. Baum and Everitt\textsuperscript{1} demonstrated that Fos-IR in the MPN is not solely a reflection of chemosensory stimulation, but depends on afferent inputs from both the MEA and CTF. Fos-IR in the MPN following intromissions was reduced following combined lesions of the MEA and CTF, but not following unilateral lesions of either the MEA or CTF. This indicates that different sensory signals are integrated in the MPN and that neural activation in the MPN may reflect this integration of sensory cues.

CONCLUSIONS

The present study demonstrates differences in neural activation following anogenital investigation versus copulation, in specific subnuclei in the male rat brain that form a heavily interconnected network. Sensory signals appear to be projected from these subnuclei to the MPN. The results of the present study indicate that the sensory signals may consist of olfactory inputs from the medial subdivision of the MEApd and BNSTpm, and genital sensory signals from the lateral part of the MEApd, subdivisions within the BNSTpm, and the SPFp. Neural activation in the MPN may therefore reflect the integration of these sensory cues, enabling the MPN to play its central role in regulating copulation.

REFERENCES


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