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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.

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

Knowledge of the neural regulation of male sexual behavior in the rat is largely based on studying copulatory behavior following electroty or chemical lesions, electrical stimulation or implantation of small quantities of steroid hormones in specific brain areas (reviewed in Sachs and Meisel4,5). 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 terminals (BNST), medial amygdala (MEA) and central tegmental field (CTF) of male rats.1,4,16,49 Hamsters16,28,29,50 and gerbils19 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 as chemosensory investigation of the female, and consummatory behaviors, i.e. mounting, intromissions and ejaculations.15,43 Previous studies in male rat,1 hamster16,28 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

Abbreviations: AOB, accessory olfactory bulb; BNST, bed nucleus of the stria terminalis; BNSTal, anterolateral part of the BNST; BNSTcm, centromedial part of the BNST; BNSTpl, posterolateral part of the BNST; BTNSTpm, postero-medial part of the BNST; CTF, central tegmental field; IR, immunoreactive, immunoreactivity; LHRH, luteinizing hormone-releasing hormone; MEA, medial amygdala; MEApd, posterodorsal part of the MEA; Ml, medial lamincus; MPN, medial preoptic nucleus; MPOA, medial preoptic area; PA, posterior nucleus of the amygdala; PBS, phosphate-buffered saline; SPPc, parvicellular part of the subparafascicular thalamic nucleus.
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.

**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. Female Wistar rats (200 g) were bilaterally ovariectomized 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 × 50 × 65 cm³) 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 inexperienced males interacted with a non-receptive anestrous female. These males displayed only chemosensory investigation in which copulation is prevented, such as tapping the vaginal region of the female, placing a female behind a screen or studying males that do not mate. Males interacting with an anestrous female have free access to the genital regions and display chemosensory investigation equivalent to males paired with estrous females.

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 heparin (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 110) 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 antisera 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 H0140987) and in sheep peroxidase-antiperoxidase (1:600 in incubation solution; Nordic 3370), each for 90 min at room temperature. Between each incubation, 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 (LHRH)-containing neurons, since copulation or exposure to a female induces release of luteinizing hormone. Therefore, all sections were

<table>
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<tr>
<th>Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<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>
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<tr>
<td>Mating experience</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Handling and exploration</td>
<td>+</td>
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Groups A–C, n=5; Groups D and E, n=4.
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 and ferrets. 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.

**Results**

**Amygdaloid complex**

In the bed nucleus of the accessory olfactory tract, postero medial cortical nucleus, antero dorsal, antero ventral and posterodorsal 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 estrous 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, 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**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aco</td>
<td>anterior commissure</td>
</tr>
<tr>
<td>cc</td>
<td>corpus callosum</td>
</tr>
<tr>
<td>CEA</td>
<td>central nucleus of the amygdala</td>
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<tr>
<td>cd</td>
<td>cerebral peduncle</td>
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<tr>
<td>fr</td>
<td>fasciculus retroflexus</td>
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<tr>
<td>fx</td>
<td>fornix</td>
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<tr>
<td>IA</td>
<td>intercalated nuclei of the amygdala</td>
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<tr>
<td>LSv</td>
<td>ventral part of the lateral septum</td>
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<tr>
<td>oc</td>
<td>optic chiasm</td>
</tr>
<tr>
<td>opt</td>
<td>optic tract</td>
</tr>
<tr>
<td>PAG</td>
<td>periaqueductal gray</td>
</tr>
<tr>
<td>PD</td>
<td>posterodorsal preoptic nucleus</td>
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<tr>
<td>PMV</td>
<td>ventral premammillary nucleus</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
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<tr>
<td>sm</td>
<td>stria medullaris</td>
</tr>
<tr>
<td>SNe</td>
<td>substantia nigra, compact part</td>
</tr>
<tr>
<td>SNr</td>
<td>substantia nigra, reticular part</td>
</tr>
<tr>
<td>st</td>
<td>stria terminalis</td>
</tr>
<tr>
<td>STN</td>
<td>subthalamic nucleus</td>
</tr>
<tr>
<td>v3</td>
<td>third ventricle</td>
</tr>
<tr>
<td>vi</td>
<td>lateral ventricle</td>
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Fos-IR neurons were scattered throughout the nucleus.

**Bed 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 naïve 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.

Preoptic 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. 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. These data, in turn, have relevance to earlier reports on the disruption of sexual behavior following lesions and the facilitatory effects of electrical stimulation. 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.
and MEA) follows consummatory behavior. Considering the differences in the distribution and number of neurons activated in the BFST versus the MEA, it can be inferred that the BFST provides more consistent and reliable evidence of the neural activity associated with consummatory behavior. Furthermore, the BFST is shown to have a higher number of neurons activated in the BFST versus the MEA, indicating a more robust consummatory response. Moreover, the BFST shows a more consistent pattern of neuronal activation, with a higher number of neurons activated in the BFST versus the MEA, suggesting a more coordinated neural response. Finally, the BFST shows a higher number of neurons activated in the BFST versus the MEA, indicating a more robust consummatory response.
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, neural activation in the MEApd 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 not 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 MEApd of sexually inexperienced males, thus implying that the distribution of the Fos induction following anogenital investigation or mating appears to be a result of sensory cues related to the test environment or to learning processes. Indeed, the MEA is indicated to play a role in learning and memory. However, the present study failed to demonstrate increased neural activation in the MEA or BNST at a result of previous experience or exposure to olfactory inputs from the mating arena alone, since there were no differences with inexperienced males placed alone in a clean test cage. In contrast, a recent report by Bressler 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. Instead, it contained the accumulated odors from the mating arena. Therefore, the olfactory cues derived from this bedding may not have been strong enough to induce Fos-IR or serve as a sexually conditioned stimulus.

Although neural activation in the medial portion of the MEApd appears to reflect activation by chemosensory cues, neural activation in the BNSTpm seems to indicate the involvement of this brain region in directing chemosensory behavior, rather than solely reflecting processing of chemosensory inputs. Disruption of olfactory inputs was reported to have no effect on c-fos expression in the BNSTpm. Fernandez-Fewell and Meredith reported that removal of the vomeronasal organs had no effect on the induction of fos-IR in the BNSTpm in male hamsters stimulated with vaginal fluid or displaying chemosensory investigation. Also, in rats, induction of Fos-IR in the BNST was not reduced as a result of lesions of the olfactory peduncle. In agreement, the present study demonstrated neural activation in the BNSTpm of sexually inexperienced males performing chemosensory investigation. Unfortunately, the results of the present study did not clearly indicate a significance increase of neural activation in experienced males following anogenital investigation, due to a large variance in the numbers of Fos-IR neurons. However, the five-fold increase in sexually experienced males following anogenital investigation was not different from that in inexperienced males.

The results of the present study demonstrating neural activation in the MEApd and BNST following anogenital investigation are consistent with an earlier report by Baum and Everitt, showing neural activation in the MEA and BNST in males that had free access to estrous females but showed no interest in mating. The paradigm used in the present study to investigate Fos-IR induced by anogenital investigation consisted of exposure to an anestrous female. The goal of using this test situation was to compare neural activation following display only of chemosensory investigation with activation resulting from copulation including chemosensory investigation. Although males will show a preference for estrous females over anestrous females, indicating that the olfactory cues derived from an estrous female are qualitatively different from an anestrous female, males will spend an equal amount of anogenital investigation of anestrous or estrous females. Therefore, the display of anogenital investigation and the resulting 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.

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 MEA, BNST, and BNSTpm. The BNSTpm is a brain area that receives ascending genital sensory input directly or indirectly from the lumbosacral spinal cord and sends projections to the MPN, PA, and MEA.
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, Fos-positive neurons were observed in male hamsters and gerbils. 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 medially 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 visceral sensory input from the internal reproductive cord that are involved in the motor aspects of copulation.

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, both of which receive genital sensory inputs from the SPFp.

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. 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 chemo- or genosensory investigation, in agreement with previous reports, but was only detected following consummatory behavior. Baum and Everitt demonstrated that Fos-IR in the MPN is not solely a reflection of chemo- or genosensory 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


(*Accepted 1 October 1996*)