Effect of the nitric oxide level in the medial preoptic area on male copulatory behavior in rats

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Sato, Yoshikazu, Hiroki Horita, Toshie Kurohata, Hideki Adachi, and Taiji Tsukamoto. Effect of the nitric oxide level in the medial preoptic area on male copulatory behavior. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R243–R247, 1998.—We investigated the influence of the extracellular nitric oxide (NO) level on male copulatory behavior. We confirmed the changes of nitrite (NO₂⁻) and nitrate (NO₃⁻) in the medial preoptic area (MPOA) by administration of the NO precursor L-arginine (L-Arg, 10 mM) or the NO synthase inhibitor N⁵-monomethyl-L-arginine (L-NMMA, 10 mM) via a dialysis probe. NO₂⁻ and NO₃⁻ were measured simultaneously by an in vivo microdialysis method coupled with the Griess reaction. L-Arg induced significant elevations of extracellular NO₂⁻ and NO₃⁻. L-NMMA significantly reduced NO₂⁻ and NO₃⁻ levels. We observed male copulatory behavior during infusion of L-Arg or L-NMMA. Administration of L-NMMA reduced the mount rate. These findings suggested that the elevation of extracellular NO in the MPOA facilitates male copulatory behavior of rats, whereas the decrease of NO reduces their copulatory behavior.

NITRIC OXIDE (NO) is becoming recognized as one of the important intracellular messengers in the brain (7, 8). It had been reported that brain NO might concern emotional and behavioral regulation (5, 25, 34). There is a possibility that brain NO is involved in regulating male rat sexual behavior. There have been a few reports concerning the role of NO in a specific brain area on male copulatory behavior, especially penile erection (21, 22). However, there are, to our knowledge, no reports that have investigated the relationship between the extracellular NO level and male copulatory behavior. Therefore, we investigated the effect of changes of the NO level in the MPOA on male copulatory behavior with the use of a new method for simultaneous quantitative measurements of nitrite (NO₂⁻) and nitrate (NO₃⁻). We observed the changes of NO₂⁻ and NO₃⁻ levels caused by administration of the NO precursor L-arginine (L-Arg) and the synthesis inhibitor N⁵-monomethyl-L-arginine (L-NMMA) via a microdialysis probe in the medial preoptic area (MPOA). MPOA is one of the most critical areas in mediating male copulatory behavior (4, 10, 19, 20), and NO synthase (NOS)-containing neurons are localized in it (2, 34). Then we evaluated male copulatory behavior during infusion of L-Arg or L-NMMA.

MATERIALS AND METHODS

Experimental animals. Male and female Wistar rats 12 wk of age (Nihon SCL, Shizuoka, Japan) were used in this study. The animal were housed under a 12:12-h light-dark cycle. Food and water were freely available. Male rats were sexually experienced. We excluded sexually inactive rats by several sexual behavior tests prior to the experiment. The females had been ovariectomized and were administered intramuscular estradiol benzoate at a daily dosage of 5 mg to induce estrus beginning from 5 days before mating, and only females showing lordosis were used in the experiment. The other details of housing conditions of the animals were described in previous reports (29, 30).

Preparation of experimental model. The stereotactic coordinates for the tip of the cannula were 0.8 mm posterior, 0.8 mm lateral, and 7 mm ventral (27). The dialysis probe was 2 mm in length and 0.22 mm OD (A-12–02; EICOM, Kyoto, Japan). The location of the tip of the probe was confirmed to be in the MPOA by histological examination. The other details of surgical procedures were described in previous reports (29, 30).

Microdialysis and measurements of NO metabolite levels. We measured extracellular NO₂⁻ and NO₃⁻ in the microdialysis system. The dialysis probe was perfused at a constant flow rate of 2 µl/min using an infusion pump (EP-60; EICOM). Samples were collected every 10 min throughout the experiment. Microdialysis samples were analyzed with an automated NO detector-high-performance liquid chromatography system (ENO-10, EICOM). NO₂⁻ and NO₃⁻ in the dialysate were separated by a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6 × 50 mm, EICOM), and NO₃⁻ was reduced to NO₂⁻ in a reduction column packed with copper-plated cadmium filings (NO-RED, EICOM). NO₂⁻ was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The separation and reduction column, and reaction coil were set at 35°C using a column oven. The absorbance of the color of the product dye at 540 nm was measured by a flow-through spectrophotometer (NOD-10, EICOM). The mobile phase was 10% methanol containing 0.15 M NaCl/NH₄Cl and 0.5 g/l Na-EDTA. It was delivered by a pump at a rate of 0.33 ml/min. The Griess reagent was 1.25% HCl containing 5 g/l sulfanilamide with 0.25 g/l N-naphthylethulenediamine. It was delivered at a rate of 0.1 ml/min. The contamination of NO₂⁻ and NO₃⁻ in Ringer solution and the reliability of the reduction column were examined in each experiment.

Schedule for measurements. We examined the effect of L-Arg or L-NMMA on NO₂⁻ and NO₃⁻. L-Arg and L-NMMA were dissolved in Ringer solution. The concentration of L-Arg or L-NMMA was 10 mM. The pH of these solutions was adjusted to 7.4. The drugs were infused into the MPOA via the microdialysis probe for 100 min. We also confirmed increments of NO₂⁻ and NO₃⁻ induced by 1 mM NMDA. NMDA was administered with the same method. L-Arg, L-NMMA, and N-methyl-D-aspartate (NMDA) were purchased from Sigma (St. Louis, MO). During a baseline sample period, the MPOA was irrigated with Ringer solution [(in mM) 147 NaCl, 2.3 CaCl₂, and 4 KCl, pH 6.0]. Six baseline samples were collected. Following baseline sample period, the perfusion of 10 mM L-Arg or L-NMMA or NMDA was started. NO₂⁻ and NO₃⁻ were measured for 100 min.
We also confirmed the interaction of L-NMMA and L-Arg. At first, 10 mM L-NMMA was infused for 60 min, followed by L-Arg infusion for 100 min. NO$_2$ and NO$_3$ levels were also measured.

The rats were placed in a plastic observation cage 30 × 30 × 35 cm (height) in size under freely moving conditions throughout all experiments.

Sexual behavior test. We observed male copulatory behavior during infusion of L-Arg or L-NMMA. Sexual behavior was observed for 10 min at 90 min after infusion was started, because the changes of NO metabolites are at a plateau at this time. The numbers of mounts with and without intromission (NI and NM, respectively) and number of ejaculations were measured for a 10-min observation period. The results were reported as the mount rate, intromission ratio, and the percentages of the rats that ejaculated. The mount rate was calculated as the mean number of NM + NI per minute, excluding the post-ejaculatory interval. The intromission ratio was calculated as NI/NM + NI.

The sexual behavior test in L-Arg and L-NMMA group was performed twice with an interval of 1 wk between tests. The rats of each group were divided into two subgroups. The first subgroup underwent the sexual behavior test with infusion of Ringer solution alone first, followed by the test with L-Arg or L-NMMA administration after 1 wk. The second subgroup was allocated L-Arg or L-NMMA first, followed by Ringer solution alone after 1 wk.

All experiments were performed in accordance with the Guidelines for Animal Experiments of Sapporo Medical University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Data analysis. The changes of NO$_2$ and NO$_3$ levels were expressed as a percentage of the six control samples. Analyses of the data on NO$_2$ and NO$_3$ were done by repeated-measures ANOVA. Data on copulatory behavior were analyzed by the Mann-Whitney U test or chi-square analysis. A P value of < 0.05 was regarded as significant.

RESULTS

Changes of extracellular NO$_2$ and NO$_3$ with infusion of drugs into the MPOA. Extracellular NO$_2$ and NO$_3$ were significantly increased by administration of L-Arg (Fig. 1). L-NMMA significantly reduced extracellular NO$_2$ and NO$_3$ levels (Fig. 2). NO$_2$ and NO$_3$ showed a plateau at 90 min after infusion of both drugs was started. NMDA significantly increased the NO$_2$ and NO$_3$ levels (Fig. 3). However, L-Arg did not elevate the NO$_2$ and NO$_3$ levels following L-NMMA infusion (Fig. 4).

Changes of copulatory behavior during infusion of L-Arg or L-NMMA in the MPOA. Administration of L-Arg significantly increased the mount rate in both groups. However, administration of L-Arg did not affect the intromission ratio or the percentage of the rats that ejaculated (Table 1). The order of administration of drugs did not affect the effects of drugs on copulatory behavior.

Administration of L-NMMA significantly reduced the mount rate and the percentages of the rats that ejaculated in both groups, but did not affect the intromission ratio (Table 2). The order of administration of drugs did not affect the effects of drugs on copulatory behavior.

DISCUSSION

NO has also been reported to be a neuronal messenger in the brain (8). Many experimental results have suggested biological roles for brain NO in neurotoxicity (7), synaptic plasticity, long-term potentiation (26, 31), synaptic plasticity, long-term potentiation (26, 31),
administration into the ventricles impaired copulation (1), and an NO synthesis inhibitor in the paraventricular nucleus (PVN) prevents apomorphine-, oxytocin-, and NMDA-induced penile erection (21, 22). There are no reports that investigated the relationship of the extracellular NO level and male copulatory behavior.

We investigated these issues in the MPOA, because there is widespread agreement that the MPOA is one of the most critical areas of the brain mediating male sexual behavior, as is the PVN (4, 10, 19, 20). Recent research has confirmed the existence of NOS-containing neurons in the MPOA. Large fibers in which NO synthesis occurs are scattered throughout the MPOA (34), and NO neurons and NMDA receptors are localized there as well (2).

We used a new method for quantitative measurements of NO2 and NO3 simultaneously using an in vivo brain microdialysis technique. We used the in vivo microdialysis method coupled with the Griess reaction. Our system produced higher sensitivity (14, 15, because of its high quality pump with less noise, even at a low flow rate than hitherto used methods (14, 15, 22, 24). The method has a high detection limit of NO2 and NO3 (0.1 pmol), which is below the physiological concentrations in the brain (0.1 pmol).

### Table 1: Copulatory behavior during infusion of L-Arg

<table>
<thead>
<tr>
<th>Group 1 (n = 13)</th>
<th>Infusion</th>
<th>Ringer</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mount rate, no./min</td>
<td>2.62 ± 1.39</td>
<td>1.61 ± 0.77</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>Intromission ratio</td>
<td>0.46 ± 0.27</td>
<td>0.47 ± 0.27</td>
<td>NS*</td>
</tr>
<tr>
<td>Ejaculation, %</td>
<td>54</td>
<td>46</td>
<td>NS†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2 (n = 14)</th>
<th>Infusion</th>
<th>Ringer</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mount rate, no./min</td>
<td>1.41 ± 0.78</td>
<td>2.45 ± 0.82</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>Intromission ratio</td>
<td>0.43 ± 0.27</td>
<td>0.48 ± 0.18</td>
<td>NS*</td>
</tr>
<tr>
<td>Ejaculation, %</td>
<td>42</td>
<td>50</td>
<td>NS†</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD (median values, range of values). L-Arg, L-arginine. *By Mann-Whitney U test; †by chi-square test.

### Table 2: Copulatory behavior during infusion of L-NMMA

<table>
<thead>
<tr>
<th>Group 1 (n = 9)</th>
<th>Infusion</th>
<th>Ringer</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mount rate, no./min</td>
<td>0.78 ± 0.91</td>
<td>2.29 ± 1.39</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>Intromission ratio</td>
<td>0.30 ± 0.30</td>
<td>0.51 ± 0.25</td>
<td>NS*</td>
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<tr>
<td>Ejaculation, %</td>
<td>22</td>
<td>44</td>
<td>P &lt; 0.01†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2 (n = 9)</th>
<th>Infusion</th>
<th>Ringer</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mount rate, no./min</td>
<td>2.13 ± 0.82</td>
<td>1.14 ± 0.75</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>Intromission ratio</td>
<td>0.39 ± 0.28</td>
<td>0.36 ± 0.25</td>
<td>NS*</td>
</tr>
<tr>
<td>Ejaculation, %</td>
<td>44</td>
<td>22</td>
<td>P &lt; 0.01†</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD (median values, range of values). L-NMMA, N2-monomethyl-L-arginine. *By Mann-Whitney U test; †by chi-square test.

and learning and memory formation (3, 36). Some reports indicated that NO in the brain is associated with regulating behaviors such as feeding (33), alcohol consumption (28), and sexual behavior (25). However, there have been a few reports about the role of NO in a specific brain area regulating behaviors. Concerning sexual behavior, it had been reported that L-NAME...
We believe that our experimental method has beneficial aspects for investigating the effect of NO in a specific brain area on behavior. Studies with general administration of drugs or using knockout mice could not identify the brain areas related to the behavioral changes. General administration of most drugs that modulate NO synthesis influence macrophage and endothelial NOS as well as neuronal NOS (nNOS) and can affect general biological systems that relate to NO. Our method of drug administration via a microdialysis probe may produce less stimulatory pressure than the microinjection method. Therefore, it might be possible to evaluate the pharmacological effect of a drug more accurately with our method.

In this study, NO\textsubscript{2} and NO\textsubscript{3} levels were elevated by L-Arg, and NO metabolites were decreased by administration of the NOS inhibitor L-NMMA via the microdialysis probe. When L-Arg was infused after infusion of L-NMMA, NO\textsubscript{2} and NO\textsubscript{3} showed no significant changes. Therefore, the changes of NO\textsubscript{2} and NO\textsubscript{3} indicated the state of NO production in the MPOA.

Moreover, NO\textsubscript{2} and NO\textsubscript{3} levels were elevated by NMDA administration. It is thought that NMDA receptor agonists increase nNOS activity (8). Therefore, NO\textsubscript{2} and NO\textsubscript{3} levels may be related to NO release from NOS-containing neurons in the MPOA. Luo et al. (18) indicated that infusion of NMDA results in a dose-dependent increase in cerebellar NO release. They concluded that this is direct evidence for NO release in vivo. Yamada and Nabeshima (35), using a microdialysis system similar to ours, indicated that NO\textsubscript{2} and NO\textsubscript{3} levels may be related to NO production in vivo.

In this experiment, the changes of mount rate were reproduced regardless of the order of administration of drugs. Therefore, we believe the changes in mount rate were due to the pharmacological effects of drugs, not the order of administration. However, the explanation of changes of mount is difficult, because mount rate might relate to sexual motivation and other factors (20). The intromission ratio is recognized as the most common and useful parameter of the male's erectile potential or penile sensitivity (20). Mount rates are also affected by the number of intromissions, because male rats pause longer after a mount with intromission than that without intromission. In this study L-Arg and L-NMMA did not affect the intromission ratio. Reduced receptivity of the female also may affect mount rate. We confirmed that the female rats showed acceptability to the male rats before the sexual behavior test. Therefore, we speculate that the NO level in the MPOA mainly modulates the mount rate through sexual motivation.

Administration of L-NMMA significantly reduced the percentage of the rats that ejaculated. Decreased occurrence of mounts and intromissions caused by L-NMMA might be related to the reduction of occurrence of ejaculation.

In the MPOA, dopamine plays an especially important role in facilitating male copulatory behavior (20). The intra-MPOA infusion of apomorphine, a mixed D\textsubscript{1}/D\textsubscript{2} dopamine receptor antagonist, increases the intromission ratio, ejaculation frequency, and frequency of reflexive erection and shortens the intromission interval (11, 13). Using microdialysis, we (30) and Hull et al. (12) have already indicated that dopamine increases significantly in the MPOA during copulation. These findings suggested that elevation of the extracellular dopamine level in the MPOA facilitates copulatory behavior in male rats, although D\textsubscript{1} and D\textsubscript{2} receptors have different effects on the behavior.

There is a possibility that NO facilitates male copulatory behavior through acceleration of dopamine release. Lorrain and Hull (16) reported that the precursor L-Arg, administered into the MPOA, increased the extracellular dopamine level. Moreover, they showed the possible role of NO in the control of dopamine release during copulation (17). They suggested that NO may play a role in control of male copulatory behavior and temperature regulation through the modulation of monoamine release. L-Glutamate elicits an intracavernous pressure increase in the MPOA (9). It increases NO production by activation of NMDA receptors. Therefore, it is possible that NO in the MPOA directly promotes penile erection, although our results suggest that NO would enhance sexual motivation. Together with these reports, our current findings support a biological role of NO in the MPOA for positive mediation of male sexual behavior.

Conclusion. Increasing the level of the NO level in the MPOA accelerates copulatory behavior of male rats, and decreasing the NO level reduces male copulatory behavior. We speculate that the NO level in the MPOA mainly modulates the mount rate through sexual motivation, although the mechanism by which NO accomplishes this is not yet well understood.

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