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# *N*-Methyl-D-aspartic acid-induced penile erection and yawning: role of hypothalamic paraventricular nitric oxide

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**Abstract**

A dose of *N*-methyl-D-aspartic acid (NMDA, 50 ng) that induces penile erection and yawning when injected into the paraventricular nucleus of the hypothalamus, increased the concentration of $NO_2^-$ from $1.10 \pm 0.28$ μM to $7.32 \pm 1.12$ μM and of $NO_3^-$ from $4.96 \pm 0.69$ μM to $10.5 \pm 1.61$ μM in the paraventricular dialysate obtained from male rats by in vivo microdialysis. $NO_2^-$ concentration was not increased by (±)-α-(amino)-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA, 100 ng) or by *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD) (100 ng), which were unable to induce these behavioral responses. *N*-Methyl-D-aspartic acid effect on $NO_2^-$ concentration, penile erection and yawning was prevented by dizolcipine (MK-801) (10–100 ng) or by the nitric oxide synthase inhibitor $N^G$-nitro-L-arginine methyl ester (20 μg), but not by the oxytocin receptor antagonist [d($CH_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin (100 ng), or by the guanylate cyclase inhibitor methylene blue (20 μg) given in the paraventricular nucleus 15 min before *N*-methyl-D-aspartic acid or by the dopamine receptor antagonist haloperidol (0.5 mg/kg) given intraperitoneally 30 min before *N*-methyl-D-aspartic acid. In contrast, the nitric oxide scavenger hemoglobin (20 μg) given in the paraventricular nucleus prevented *N*-methyl-D-aspartic acid-induced $NO_2^-$ concentration increase, but was unable to prevent penile erection and yawning. The results suggest that *N*-methyl-D-aspartic acid induces penile erection and yawning by increasing nitric oxide synthase activity in the paraventricular nucleus of the hypothalamus, possibly in the cell bodies of oxytocinergic neurons projecting to extra-hypothalamic brain areas and mediating these behavioral responses.

*Keywords:* NMDA (*N*-methyl-D-aspartate); Nitric oxide (NO); Dopamine; Oxytocin; Penile erection; Yawning; Paraventricular nucleus of the hypothalamus; (Rat)

## 1. Introduction

Penile erection and yawning are two different behavioral patterns that often occur concomitantly in physiological and experimental conditions (Bertolini and Gessa, 1981; Holmgren et al., 1985; Argiolas and Gessa, 1991). Although the importance of penile erection in reproduction does not need to be stressed, this response can be observed also in other contexts, such as manipulation of the genitalia, erotic fantasies and sleep in humans. Depending on the context in which penile erection occurs, different neural and/or humoral mechanisms may participate in its regulation (see Dail, 1987; Lue and Tanagho, 1987; Ignarro, 1992; Marson and McKenna, 1992; Hull et al., 1994; Meisel and Sachs, 1994; Argiolas and Melis, 1995; Melis and Argiolas, 1995b). As to the physiological significance of yawning, this act alone or associated with stretching is considered an ancestral vestige surviving through evolution that subserves the purpose of arousal (Bertolini and Gessa, 1981) although its role is far from being clarified (Provine et al., 1987). Among substances that induce both these responses the best known are dopamine receptor agonists (Melis and Argiolas, 1995a), oxytocin (Argiolas and Gessa, 1991), adrenocorticotropin and related peptides (Bertolini and Gessa, 1981), and serotonin receptor agonists that act mainly on the 5-$HT_{1C}$ receptor subtype (see Stancampiano et al., 1994 and references therein). Recently, *N*-methyl-D-aspartic acid (NMDA) was also found to be able to induce penile erection and yawning when injected in nanogram amounts in the paraventricular nucleus of the hypothalamus (Roeling et al., 1991; Melis et al., 1994a). These NMDA responses are apparently mediated by the stimulation of the excitatory amino acid receptors of the NMDA subtype

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since they were prevented by dizolcipine (MK-801), a non-competitive antagonist of these receptors, and were not induced by (±)-α-(amino)-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) or by *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD), agonists of the AMPA and metabotropic receptor subtype, respectively (see Monaghan et al., 1989). These behavioral responses induced by NMDA, like those induced by dopamine agonists and oxytocin, are apparently mediated by the activation of central oxytocinergic transmission, being prevented by the central administration of oxytocin receptor antagonists (see Melis et al., 1994a; Argiolas and Melis, 1995; Melis and Argiolas, 1995a).

Recently we found that the putative neurotransmitter/neuromodulator nitric oxide (NO) (see Ignarro, 1990; Snyder, 1992; Moncada and Higgs, 1993; Schuman and Madison, 1994) is involved in penile erection and yawning induced by *N*-methyl-D-aspartic acid, apomorphine, oxytocin and 5-$HT_{1C}$ receptor agonists. In particular, the above compounds (except 5-$HT_{1C}$ receptor agonists, Melis et al., 1995) seem to induce these behavioral responses by activating NO synthase in the paraventricular nucleus of the hypothalamus, which in turn leads to the activation of central oxytocinergic transmission (see above). Indeed NO synthase inhibitors prevent NMDA-, apomorphine- and oxytocin-induced penile erection and yawning when injected into the paraventricular nucleus of the hypothalamus (Melis et al., 1994a,b; Argiolas and Melis, 1995), and NO donors injected into the paraventricular nucleus induce penile erection and yawning that are indistinguishable from those induced by the above compounds, since these responses are prevented by the oxytocin receptor antagonist [$d(CH_2)_5$,$Tyr(Me)^2$, $Orn^8$]vasotocin given into the lateral ventricles (i.c.v.) (Melis and Argiolas, 1995b).

As to the possible activation by NMDA of NO synthase in this hypothalamic nucleus, which would in turn induce penile erection and yawning by activating oxytocinergic transmission, it is noteworthy that the paraventricular nucleus of the hypothalamus contains very high levels not only of NO synthase in different neuronal populations, including the cell bodies of oxytocinergic neurons (Bredt et al., 1990; Vincent and Kimura, 1992; Southam and Garthwaite, 1993; Calka and Block, 1993; Torres et al., 1993; Sanchez et al., 1994), but also of glutamic acid in presynaptic boutons that impinge on magnocellular and parvocellular neurons (Van Den Pol, 1991). Furthermore, NMDA induces a $Ca^{2+}$ influx that has been associated already with the activation of NO synthase in several brain areas (Garthwaite et al., 1988; Bredt and Snyder, 1989; Luo et al., 1993; Schuman and Madison, 1994). In order to provide definite evidence that NMDA induces penile erection and yawning by activating NO synthase in the paraventricular nucleus of the hypothalamus, we studied the effect of a dose of NMDA that induces penile erection and yawning on the production of NO in the paraventricular nucleus of the hypothalamus in vivo. This was achieved by measuring the concentration of the reaction products of newly formed NO with $O_2$, $NO_2^-$ and $NO_3^-$, which represent an indirect but reliable indicator of NO production in

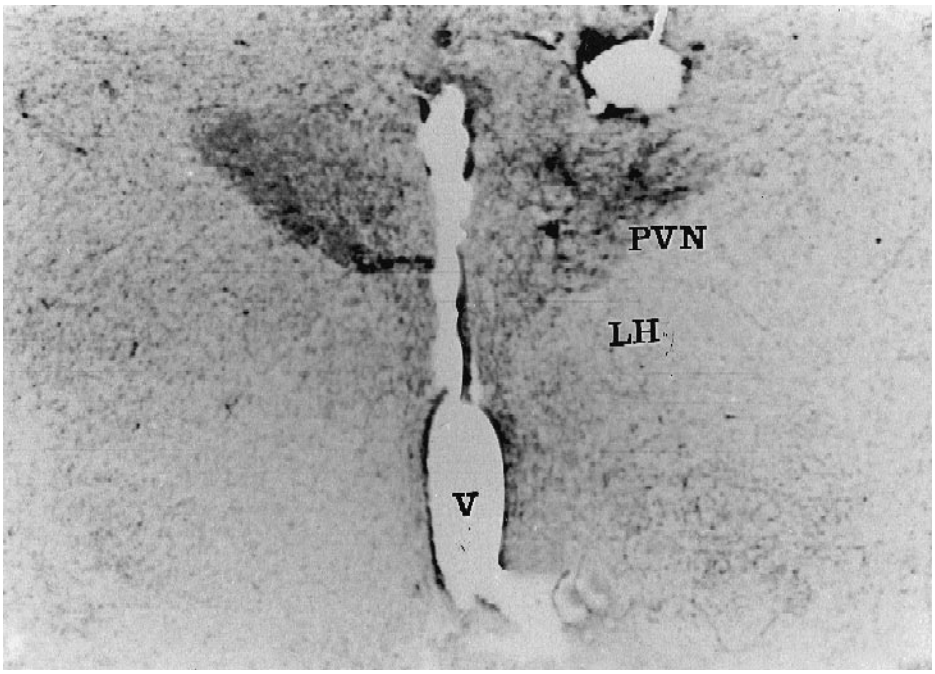


Fig. 1. Example of a Neutral Red-stained transverse brain section of a NMDA-treated rat showing the position of the tip of the microdialysis probe plus the cannula in the paraventricular nucleus of the hypothalamus. PVN, paraventricular nucleus of the hypothalamus; LH, lateral hypothalamus; V, third ventricle.

vivo (Ignarro, 1992; Luo et al., 1993; Ohta et al., 1994; Melis et al., 1996), in the dialysate collected from a vertical microdialysis probe implanted into the paraventricular nucleus. The effect of drugs which interfere with NO actions and with other neuronal systems involved in the expression of penile erection and yawning at paraventricular level on NMDA responses was also investigated.

## 2. Materials and methods

### 2.1. Animals

Male Sprague-Dawley rats (200–220 g) (Charles River, Como, Italy) were used in all the experiments. Animals were caged in groups of 4–6 at 24°C, humidity 60%, lights on from 07:00 to 19:00 h with water and standard laboratory food ad libitum. The experiments were performed between 09:00 and 13:00 h.

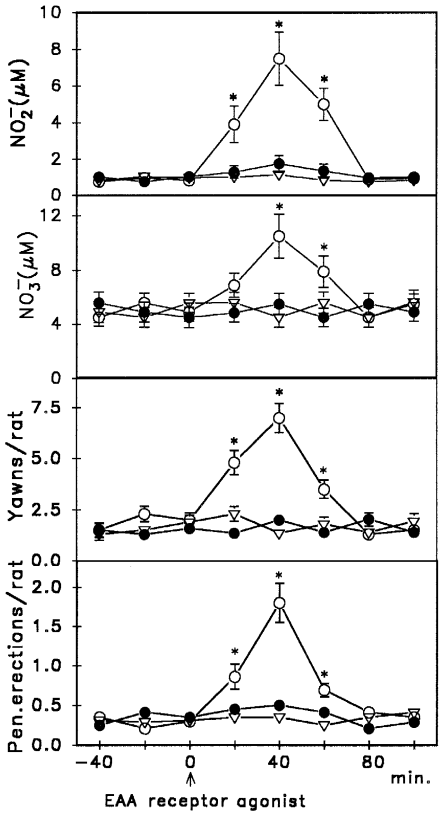


Fig. 2. Effect of the excitatory amino acid (EAA) receptor agonists NMDA, AMPA and ACPD on basal $NO_2^-$ and $NO_3^-$ concentration in the paraventricular dialysate, penile erection and yawning in freely moving male rats. Rats were placed individually into a Plexiglas cage and perfused with Ringer solution as described in Section 2. NMDA (50 ng, ○), AMPA (100 ng, ●) or ACPD (100 ng, ▽) was given in the paraventricular nucleus in a volume of 0.3 μl of saline the first time after a 120 min equilibration period of the probe with the perfusion buffer (time = 0). The perfusion rate was 2 μl/min during the experiment. Aliquots of 40 μl were collected every 20 min and analyzed for $NO_2^-$ and $NO_3^-$ content. During perfusion, the animals were observed in order to count penile erection and yawning episodes. Each value is the mean ± S.E.M. of 7 rats. * $P < 0.01$ with respect to pretreatment values (negative times) (one-way ANOVA followed by Duncan's multiple range test).

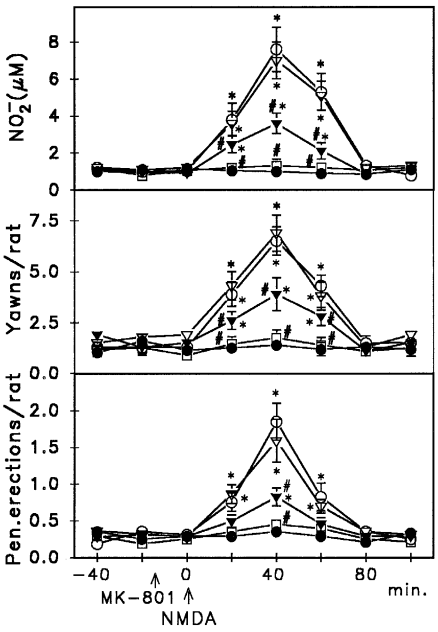


Fig. 3. Effect of MK-801 on the increase of $NO_2^-$ concentration in the paraventricular dialysate, penile erection and yawning induced by NMDA: dose–response curve. MK-801 was given 15 min before NMDA (50 ng/0.3 μl in the paraventricular nucleus). (●) MK-801-treated rats; (○) NMDA-treated rats; (▽) 10 ng MK-801 + NMDA-treated rats; (▼) 50 ng MK-801 + NMDA-treated rats; (□) 100 ng MK-801 + NMDA-treated rats. The other experimental conditions were identical to those reported in the legend to Fig. 2. Each value is the mean ± S.E.M. of 6 rats. * $P < 0.01$ with respect to pretreatment values: # $P < 0.01$ with respect to the corresponding values of NMDA-treated rats (one-way ANOVA followed by Duncan's multiple range test).

### 2.2. Drugs and peptides

$N^G$-Nitro-L-arginine methyl ester (L-NAME), rat hemoglobin, methylene blue, sulfanilamide and *N*-(1-naphthyl)-ethylenediamine were purchased from Sigma (St. Louis, MO, USA), (+)-dizolcipine ((+)-MK-801), NMDA (*N*-methyl-D-aspartic acid), (±)-α-(amino)-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD) from Research Biochemicals International (Natick, MA, USA), [d$(CH_2)_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin from Peninsula Europe (St. Helens, UK). Haloperidol was kindly provided by Janssen Pharmaceuticals (Beerse, Belgium). Since commercial hemoglobins are usually a mixture of reduced and oxidized hemoglobins that have different affinities for nitric oxide, rat hemoglobin was reduced with sodium dithionite, dialyzed against distilled water, divided in aliquots and stored at −20°C, as previously described (Martin et al., 1985). All the other reagents were of the highest available purity.

### 2.3. Microinjections in the paraventricular nucleus of the hypothalamus and in vivo microdialysis

In order to perform microinjections and microdialysis in the paraventricular nucleus of the hypothalamus of the

same animal, microdialysis probes with approximately 1 mm of free surface for dialysis, using a loop flow design, were prepared as already described (Melis et al., 1996), except that an infusion cannula made with fused capillary silica tubing ending adjacent to the U-shaped membrane was glued to the microdialysis probe with epoxy resin. The modified probes were then implanted stereotaxically (David Kopf Instruments, USA) in the paraventricular nucleus of the hypothalamus (coordinates 0.2 mm anterior to the bregma, 0.4 mm lateral to the midline, and 7.3 mm vertical from the dura) (Pellegrino and Cushman, 1971) under chloral hydrate anesthesia, 2 days before the experiments. Each rat was used only once. The probes were perfused with Ringer's solution, containing 147 mM NaCl, 3 mM KCl and 1.2 mM $CaCl_2$, pH 6.5, at a constant flow rate of 2 μl/min using a Stoelting 200 microsyringe pump. After a 2-h equilibration period, the dialysate was collected every 20 min (in fractions of 40 μl) in polyethylene tubing loops and transferred in polyethylene tubes at a temperature of 10–15°C for the determination of $NO_2^-$ and $NO_3^-$ as described below. NMDA, AMPA or ACPD was injected through the attached cannula in a volume of 0.3 μl of Ringer's solution over a period of 2 min. Controls received the same volume of Ringer's solution. Ringer's solution (0.3 μl) alone or containing MK-801, $N^G$-nitro-L-arginine methyl ester (L-NAME), [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin, methylene blue or hemoglobin was injected into the paraventricular nucleus over a period of 2 min, 15 min before NMDA.

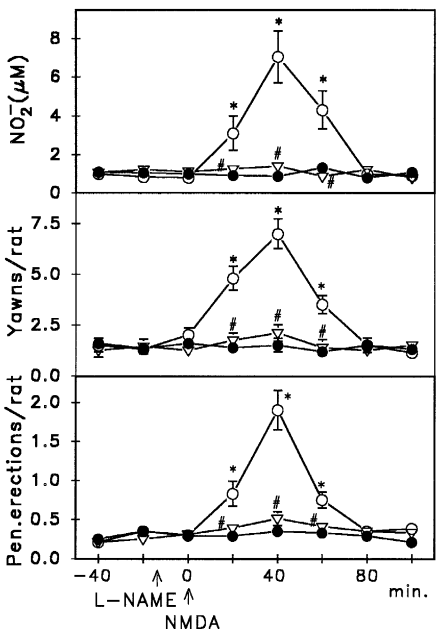


Fig. 4. Effect of L-NAME on the increase of $NO_2^-$ concentration in the paraventricular dialysate, penile erection and yawning induced by NMDA. L-NAME (20 μg) was given in the paraventricular nucleus 15 min before NMDA (20 μg in 0.3 μl). (●) L-NAME-treated rats; (○) NMDA-treated rats; (▽) L-NAME + NMDA-treated rats. The other experimental conditions were identical to those reported in the legend to Fig. 2. Each value is the mean ± S.E.M. of 6 rats. * $P < 0.01$ with respect to pretreatment values; # $P < 0.01$ with respect to the corresponding values of NMDA-treated rats (one-way ANOVA followed by Duncan's multiple range test).

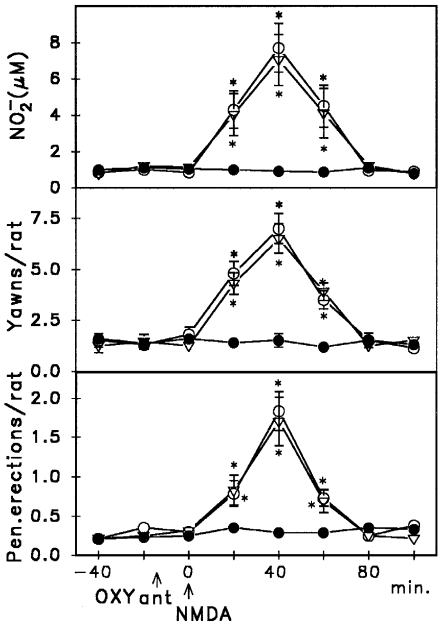


Fig. 5. Effect of [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin on the increase of $NO_2^-$ concentration in the paraventricular dialysate, penile erection and yawning induced by NMDA. [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]Vasotocin (100 ng/0.3 μl) was given in the paraventricular nucleus 15 min before NMDA (50 ng/0.3 μl in the paraventricular nucleus). (●) [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin-treated rats; (○) NMDA-treated rats; (▽) [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin + NMDA-treated rats. The other experimental conditions were identical to those reported in the legend to Fig. 2. Each value is the mean ± S.E.M. of 6 rats. * $P < 0.01$ with respect to pretreatment value (one-way ANOVA followed by Duncan's multiple range test).

### 2.4. Systemic treatments

Haloperidol was dissolved with a drop of glacial acetic acid, diluted with saline (final pH = 5.5) and injected intraperitoneally (i.p.) in a volume of 0.5 ml/100 g body weight 30 min before NMDA.

### 2.5. Determination of $NO_2^-$ and $NO_3^-$ concentration

$NO_2^-$ concentration in the paraventricular dialysate was determined by a modification of the Griess reaction as already described (Melis et al., 1996). Briefly, $NO_2^-$ in the dialysate was used for the diazotization of sulfanilamide and subsequent coupling to *N*-(1-naphthyl)-ethylene-diamine. The azo dye was then quantified by high-pressure liquid chromatography (HPLC) from its absorbance at 546 nm with a Waters LC Module I chromatograph equipped with a UV 486 detector, a WISP 715 autoinjector and a 0.4 × 15 cm Novapak C18 column (Waters Associates, Milford, MA, USA). The sensitivity of the assay was 0.1 μM equivalent to about 0.3 ng of $NaNO_2$ in 40 μl of dialysate and the response was found to be linear with

increasing concentrations of $NO_2^-$ up to 25 μM. For the determination of $NO_3^-$ in the dialysate, $NO_3^-$ was previously reduced to $NO_2^-$ with copper-cadmium by a modification of the method described by Cortas and Wakid (1990), as already described (Melis et al., 1996). The determination of total $NO_2^-$ was then performed as described above and the amount of $NO_3^-$ was then calculated by subtracting that of $NO_2^-$ found in the aliquot of dialysate without copper-cadmium reduction. The sensitivity of the method was 3 μM (10 ng of $NaNO_3$ in 40 μl of dialysate) and the response was linear with $NO_3^-$ up to 30 μM.

### 2.6. Behavioral studies

Rats were placed individually in Plexiglas cages (30 × 30 × 30 cm). After a 30 min habituation period, the microdialysis probe was connected via polyethylene tubing to a 10 μl Hamilton microsyringe driven by a Stoelting microsyringe pump on one end and to the polyethylene collecting loop on the other end. The cannula for paraventricular injections was also connected to a 10 μl Hamilton microsyringe driven by a microinfusion pump via polyethylene tubing. After a 2-h equilibration period of perfusion of the dialysis probe with Ringer's solution, NMDA, AMPA or ACPD was given in the paraventricular nucleus over a 2 min period. In those experiments in which haloperidol was used, this was given i.p. 30 min before NMDA. When

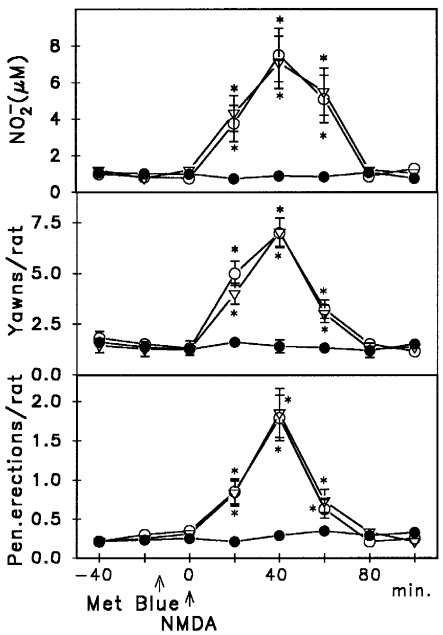


Fig. 6. Effect of methylene blue on the increase of $NO_2$ concentration in the paraventricular nucleus dialysate, penile erection and yawning induced by apomorphine. Methylene blue (20 μg/0.3 μl) was given in the paraventricular nucleus 15 min before NMDA (50 ng/0.3 μl in the paraventricular nucleus). (●) Methylene blue-treated rats; (○) NMDA-treated rats; (▽) methylene blue + NMDA-treated rats. The other experimental conditions were identical to those reported in the legend to Fig. 2. Each value is the mean ± S.E.M. of 7 rats. * $P < 0.01$ with respect to pretreatment values (one-way ANOVA followed by Duncan's multiple range test).

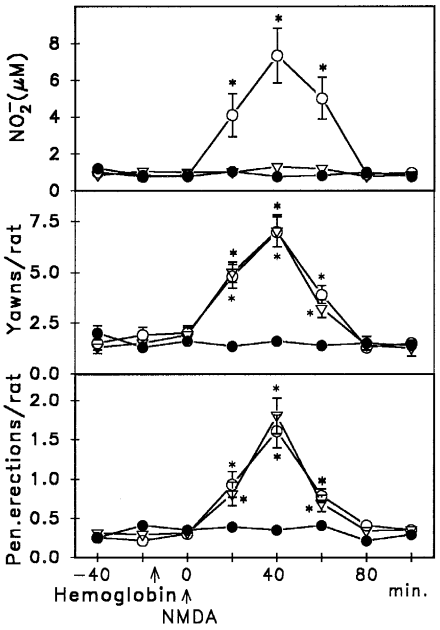


Fig. 7. Effect of hemoglobin on the increase of $NO_2^-$ concentration in the paraventricular dialysate, penile erection and yawning induced by NMDA. Hemoglobin (20 μg/0.3 μl) was given in the paraventricular nucleus 15 min before NMDA (50 ng/0.3 μl in the paraventricular nucleus). (●) Hemoglobin-treated rats; (○) NMDA-treated rats; (▽) hemoglobin + NMDA-treated rats. The other experimental conditions were identical to those reported in the legend to Fig. 2. Each value is the mean ± S.E.M. of 6 rats. * $P < 0.01$ with respect to pretreatment values; # $P < 0.01$ with respect to the corresponding values of NMDA-treated rats (one-way ANOVA followed by Duncan's multiple range test).

MK-801, L-NAME, methylene blue, hemoglobin or [d($CH_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin was given, these were injected into the paraventricular nucleus over a 2 min period 15 min before NMDA. After treatments, rats were observed for the entire duration of the experiment to replace filled loops with empty ones every 20 min and to count penile erection and yawning episodes.

### 2.7. Histology

At the end of the experiments the animals were killed by decapitation, the brains were immediately removed and stored in 2% aqueous formaldehyde for 10–12 days. To localize the position of the probe tip, 50 μm transverse brain sections were prepared by means of a freezing microtome, stained with Neutral Red and inspected on a phase-contrast microscope. The site of the probe tip was localized by following the probe tract through a series of brain sections. Only those animals found to have the probe tip positioned correctly in the paraventricular nucleus of the hypothalamus (see Fig. 1) were considered for the statistical evaluation of the results.

### 2.8. Statistics

Statistical evaluation of the results was performed by analysis of variance (one-way ANOVA), followed by Dun-

can's multiple range test. A $P < 0.05$ was considered significant (Tallarida and Murray, 1986).

## 3. Results

### 3.1. Effect of NMDA, AMPA and ACPD injected into the paraventricular nucleus of the hypothalamus on paraventricular NO production, penile erection and yawning

NMDA (50 ng), but not AMPA (100 ng) or ACPD (100 ng), injected into the paraventricular nucleus after a 2 h equilibration period, increased the concentration of $NO_2^-$ from $1.10 \pm 0.28$ μM to $7.32 \pm 1.12$ μM and of $NO_3^-$ from $4.96 \pm 0.69$ μM to $10.5 \pm 1.61$ μM in the paraventricular dialysate ($P < 0.01$). The increase was already evident 20 min after the injection, reached its maximum at 40 min and disappeared 80 min later (Fig. 2). Most important, the $NO_2^-$ and $NO_3^-$ increase induced by NMDA was associated with the appearance of penile erection and yawning episodes, which started 10–12 min after NMDA injection and lasted for 50–60 min ($P < 0.01$). In contrast, AMPA and ACPD, which were ineffective in increasing $NO_2^-$ and $NO_3^-$ concentration ($P > 0.1$, not significant), were also ineffective in inducing penile erection and yawning ($P > 0.1$, not significant) (Fig. 2).

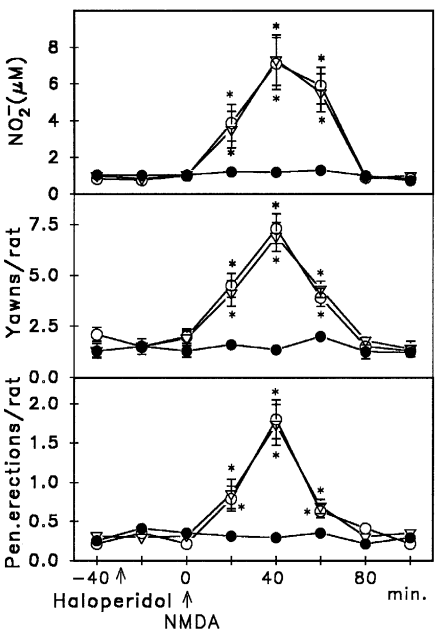


Fig. 8. Effect of haloperidol on the increase of $NO_2^-$ concentration in the paraventricular dialysate, penile erection and yawning induced by NMDA. Haloperidol (0.5 mg/kg i.p.) was given 30 min before NMDA (50 ng/0.3 μl in the paraventricular nucleus). (●) Haloperidol-treated rats; (○) NMDA-treated rats; (▽) haloperidol + NMDA-treated rats. The other experimental conditions were identical to those reported in the legend to Fig. 2. Each value is the mean ± S.E.M. of 8 rats. * $P < 0.01$ with respect to pretreatment values (one-way ANOVA followed by Duncan's multiple range test).

### 3.2. Effect of MK-801 on NMDA-induced increase of $NO_2^-$ concentration, penile erection and yawning

The effect of NMDA on $NO_2^-$ concentration in the paraventricular dialysate was prevented dose-dependently by MK-801 (10–100 ng) injected into the paraventricular nucleus 15 min before NMDA, which prevented also penile erection and yawning. A 50% prevention of either NMDA response was found with 50 ng, while a complete prevention was usually obtained with 100 ng of MK-801. At the highest dose used, the non-competitive NMDA receptor antagonist was unable alone to modify basal $NO_2^-$ concentration in the paraventricular dialysate, penile erection or yawning (Fig. 3), nor did it induce any gross behavioral change.

### 3.3. Effect of L-NAME on NMDA-induced increase of $NO_2^-$ concentration, penile erection and yawning

NMDA effects on $NO_2^-$ concentration in the paraventricular dialysate, penile erection and yawning were also prevented by L-NAME (20 μg) injected into the paraventricular nucleus 15 min before NMDA (Fig. 4). At the dose used, the NO synthase inhibitor was unable alone to modify basal $NO_2^-$ concentration in the paraventricular dialysate, penile erection or yawning (Fig. 4), nor did it induce any gross behavioral change.

### 3.4. Effect of [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin on NMDA-induced increase of $NO_2^-$ concentration, penile erection and yawning

In contrast to MK-801 and L-NAME, [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin (100 ng) injected into the paraventricular nucleus 15 min before NMDA was unable to prevent NMDA-induced increase of $NO_2^-$ concentration in the paraventricular dialysate. The nonapeptide oxytocin receptor antagonist was also ineffective on penile erection and yawning induced by NMDA (Fig. 5). At the dose used, [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin was unable alone to modify basal $NO_2^-$ concentration in the paraventricular dialysate, penile erection or yawning (Fig. 5).

### 3.5. Effect of methylene blue on NMDA-induced increase of $NO_2^-$ concentration, penile erection and yawning

Like [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin, methylene blue (20 μg) injected into the paraventricular nucleus 15 min before NMDA was unable to prevent NMDA-induced increase of $NO_2^-$ concentration in the paraventricular dialysate, penile erection and yawning. At the dose used, the putative inhibitor of guanylate cyclase was unable alone to modify basal $NO_2^-$ concentration in the paraventricular dialysate, penile erection or yawning (Fig. 6).

### 3.6. Effect of hemoglobin on NMDA-induced increase of $NO_2^-$ concentration, penile erection and yawning

In contrast to [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin and methylene blue, hemoglobin (20 μg) injected into the paraventricular nucleus 15 min before NMDA prevented NMDA-induced increase of $NO_2^-$ concentration in the paraventricular dialysate, but not penile erection and yawning. At the dose used, the NO scavenger was unable alone to modify basal $NO_2^-$ concentration in the paraventricular dialysate, penile erection or yawning (Fig. 7).

### 3.7. Effect of haloperidol on NMDA-induced increase of $NO_2^-$ concentration, penile erection and yawning

Haloperidol (0.5 mg/kg i.p.) given 30 min before NMDA was unable to modify NMDA-induced increase of $NO_2^-$ concentration in the paraventricular dialysate, penile erection and yawning. At the dose used, the dopamine receptor antagonist was unable alone to modify basal $NO_2^-$ concentration in the paraventricular dialysate, penile erection or yawning (Fig. 8).

## 4. Discussion

The present results show that NMDA, but not AMPA or ACPD, when injected into the paraventricular nucleus of the hypothalamus at a dose that induces penile erection and yawning increases basal $NO_2^-$ and, but to a lesser extent, $NO_3^-$ concentration in the paraventricular dialysate of male rats. The findings are in line with the hypothesis that NMDA induces penile erection and yawning by activating NMDA receptors, whose stimulation is coupled to the activation of NO synthase in the paraventricular nucleus (Melis et al., 1994c; Argiolas and Melis, 1995). Accordingly, NMDA-induced $NO_2^-$ increase was prevented dose dependently by MK-801, a potent non-competitive antagonist of NMDA receptors (Monaghan et al., 1989), that prevented also penile erection and yawning (Melis et al., 1994c, and the present results). As to the mechanism by means of which the stimulation of NMDA receptors activates NO synthase in the paraventricular nucleus, the activation of this enzyme, that in neurons is $Ca^{2+}$ calmodulin-dependent (see Snyder, 1992; Schuman and Madison, 1994), might be secondary to the increased influx of $Ca^{2+}$ ions that occurs through the $Ca^{2+}$ channel coupled to NMDA receptors (see Monaghan et al., 1989) possibly located in the cell bodies of oxytocinergic neurons projecting to extrahypothalamic brain areas and mediating penile erection and yawning (Melis et al., 1994a,c). A similar mechanism has been suggested to explain the activation by NMDA of NO synthase in cultured cerebellar granular cells (Bredt and Snyder, 1989) and in pyramidal hippocampal neurons (Garthwaite et al., 1988). In agreement with the above hypothesis, the increase of $NO_2^-$ and $NO_3^-$ concentration in the paraventricular dialysate induced by NMDA found in this study, like that induced by dopamine agonists and by oxytocin (Melis et al., 1996, 1997) would reflect almost exclusively an increased conversion of L-arginine to NO that is in turn oxidized mainly to $NO_2^-$ and, to a lesser extent, to $NO_3^-$, as found in other biological fluids not containing blood cells (Marletta et al., 1988; Bredt and Snyder, 1989; Ignarro, 1990; Luo et al., 1993; Ohta et al., 1994; Butler et al., 1995). Indeed the NMDA-induced increase of $NO_2^-$ concentration is also prevented by L-NAME, a potent inhibitor of NO synthase (Rees et al., 1990) given in the paraventricular nucleus, which prevents also penile erection and yawning. The NMDA-induced $NO_2^-$ increase in the paraventricular dialysate was also prevented by hemoglobin, a potent NO scavenger (Murad et al., 1978; Gruetter et al., 1981). However, the above interpretation is complicated by the inability of hemoglobin to prevent NMDA-induced penile erection and yawning. A possible explanation for this discrepancy is that NO formed by NMDA stimulation of NMDA receptors in the paraventricular nucleus of the hypothalamus acts as an intracellular messenger in the neurons in which it is formed rather than an extracellular transmitter, to induce these behavioral responses (Melis and Argiolas, 1995b). In fact, hemoglobin would be expected to scavenge only extracellular and not intracellular NO, because it is unable to cross cell membranes because of its high molecular weight. However, this does not rule out the possibility that NO released out from those cells by which it is produced, acts as an extracellular transmitter and is involved for instance in other hypothalamic effects of NMDA (for a review of NO signalling in the hypothalamus see Amir, 1995).

The inability of [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin, a potent oxytocin receptor antagonist (Bankowski et al., 1980), to prevent NMDA-induced $NO_2^-$ increase in the paraventricular dialysate as well as penile erection and yawning when injected into the paraventricular nucleus, found in this study, is not in contrast with the hypothesis that NMDA induces these behavioral responses by activating central oxytocinergic transmission, as recalled above. In fact, if one assumes that NMDA induces penile erection and yawning by acting on its own receptors located in the cell bodies of oxytocinergic neurons projecting to extrahypothalamic brain areas (i.e., the hippocampus and/or the spinal cord) (Melis et al., 1992; Argiolas and Melis, 1995), the oxytocin receptor antagonist would be expected to act in the brain areas where oxytocin is released, that is after NO formation at sites distant from the paraventricular nucleus of the hypothalamus. Accordingly, [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin injected into the lateral ventricles was able to prevent penile erection and yawning induced by NMDA (Melis et al., 1994a). A similar interpretation was used to explain the failure of [d(CH$_2$)$_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin to prevent the $NO_2^-$ increase in the paraventricular dialysate induced by

dopamine agonists, which also induce penile erection and yawning by activating oxytocinergic transmission in the paraventricular nucleus of the hypothalamus, despite its ability to prevent the behavioral responses (Melis et al., 1996).

The present results show also that the dopamine receptor antagonist haloperidol is unable to prevent NMDA effects on $NO_2^-$ concentration, penile erection and yawning, despite its ability to prevent these responses when induced by dopamine agonists (Melis et al., 1996). This is in agreement with previous studies showing that both glutamic acid and dopamine act before oxytocin in the paraventricular nucleus through the stimulation of specific receptors for the induction of these behavioral responses (Argiolas and Gessa, 1991; Melis et al., 1992; Argiolas and Melis, 1995; Melis and Argiolas, 1995a). In fact, although dopamine agonists and NMDA increase NO synthase in the same oxytocinergic neurons mediating penile erection and yawning, the selective blockade of dopamine receptors would prevent dopamine agonist- but not NMDA-induced responses (Melis et al., 1995).

Taken together, the ineffectiveness of [d$(CH_2)_5$,Tyr(Me)$^2$,Orn$^8$]vasotocin and haloperidol to prevent NMDA responses is in line with previous findings showing that NMDA does not induce penile erection and yawning by releasing dopamine from incerto-hypothalamic dopaminergic nerve endings or oxytocin from oxytocinergic dendrites, respectively, in the paraventricular nucleus, which would have in turn activated oxytocinergic neurons to induce the behavioral responses. Accordingly, excitatory amino acid receptor antagonists, including MK-801, injected into the paraventricular nucleus were unable to prevent penile erection and yawning induced by dopamine agonists or by oxytocin (Melis et al., 1993).

As to the mechanism by means of which NO endogenously formed in the paraventricular nucleus of the hypothalamus by the stimulation of NMDA receptors activates central oxytocinergic transmission to induce penile erection and yawning, only some speculation is possible at present. Guanylate cyclase is the most well known target of NO and a NO-cyclic guanosine 3′:5′-monophosphate (cGMP) signalling pathway has been characterized in several brain areas (Garthwaite et al., 1988; Bredt and Snyder, 1989; Snyder, 1992; Schuman and Madison, 1994). However, the failure of methylene blue, a putative guanylate cyclase inhibitor (Murad et al., 1978; Gruetter et al., 1981), to prevent the $NO_2^-$ increase in the paraventricular dialysate induced by NMDA as well as penile erection and yawning when injected into the paraventricular nucleus, suggests that guanylate cyclase and guanosine 3′:5′-cyclic monophosphate (cGMP) are not involved at the paraventricular level in the induction of penile erection and yawning by NMDA. This finding is in line with other experimental evidence suggesting that guanylate cyclase is not involved at the paraventricular level in the control of penile erection and yawning induced by dopamine agonists, oxytocin and NMDA itself (Melis et al., 1994b,c). Accordingly, the injection of a stable cGMP analog (e.g., 8-bromo-cGMP) into the paraventricular nucleus, unlike that of nitric oxide donors, was unable to induce penile erection and yawning (Melis and Argiolas, 1995b). In this regard, it is noteworthy that other targets of NO have been identified in addition to guanylate cyclase (for a review of other NO targets see Schuman and Madison, 1994). Nevertheless, since methylene blue given i.c.v. was able to prevent these behavioral responses induced by NMDA (Melis et al., 1994c), the possibility that guanylate cyclase is involved in the control of penile erection and yawning in sites distant from the paraventricular nucleus of the hypothalamus cannot be ruled out. This explanation should be considered with caution, since methylene blue has been reported to inhibit directly NO synthase rather than guanylate cyclase, at least in vitro (Mayer et al., 1993). However, the inability of methylene blue injected directly into the paraventricular nucleus to decrease NMDA-stimulated $NO_2^-$ and $NO_3^-$ concentration, does not support such a possibility in our experimental conditions.

In conclusion, the present results show that NMDA injected into the paraventricular nucleus of the hypothalamus at a dose that induces penile erection and yawning, increases the concentration of $NO_2^-$ and $NO_3^-$ in the paraventricular dialysate. Since $NO_2^-$ and $NO_3^-$ concentration in extracellular fluids is a reliable index of the activity of NO synthase (Ignarro, 1990; Luo et al., 1993; Ohta et al., 1994; Melis et al., 1996, 1997) these results provide further evidence that NO plays an important role in the control of these behavioral responses at the level of this hypothalamic nucleus.

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