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Different effects of ω-conotoxin on penile erection, yawning and paraventricular nitric oxide in male rats

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Abstract

A dose of apomorphine or oxytocin that induces penile erection and yawning increases nitric oxide production in the paraventricular nucleus of the hypothalamus, as determined by the increase in NO_2^- and NO_3^- concentration induced by these substances in the paraventricular dialysate obtained from male rats. All the above responses were prevented by a dose of ω-conotoxin-GVIA as low as 5 ng. This potent inhibitor of N-type Ca^{2+} channels was injected into the paraventricular nucleus 15 min before apomorphine (50 ng) or oxytocin (10 ng). In contrast, ω-conotoxin was ineffective when the above responses were induced by *N*-methyl-D-aspartic acid (50 ng). The peptide toxin (5 ng) was also ineffective on the penile erection and yawning induced by the nitric oxide donors sodium nitroprusside (50 μg) or hydroxylamine (50 μg), injected into the paraventricular nucleus. The present results suggest that ω-conotoxin-sensitive Ca^{2+} channels are involved in the activation of nitric oxide synthase, penile erection and yawning induced by apomorphine and oxytocin, but not by *N*-methyl-D-aspartic acid, at the paraventricular level.

Keywords: Penile erection; Yawning; Ca^{2+} channel; ω-Conotoxin-GVIA; Apomorphine; Oxytocin; NMDA (*N*-Methyl-D-aspartate); Nitric oxide (NO) donor; Paraventricular nucleus; (Rat)

1. Introduction

Penile erection and yawning are two different behavioural patterns that often occur concomitantly under different physiological and experimental conditions (Holmgren et al., 1985; Argiolas and Gessa, 1991; Meisel and Sachs, 1994; Argiolas and Melis, 1995, 1998). While the importance of penile erection in reproduction does not need to be stressed, it is pertinent to recall that yawning is considered an ancestral vestige that subserves the purpose of arousal, although its role is far from being clarified (see Argiolas and Melis, 1998). Dopamine receptor agonists, such as apomorphine, the neurohypophyseal peptide, oxytocin and *N*-methyl-D-aspartic acid (NMDA), a selective agonist of the excitatory amino acid receptor of the Ca^{2+} channel-coupled NMDA subtype (Monaghan et al., 1989), are among the most potent inducers of this symptomatology in male rats. In particular, all these substances induce penile erection and yawning when injected in nanogram amounts into the paraventricular nucleus of the hypothalamus (for review see Argiolas and Melis, 1995, 1998 and references therein). Several lines of experimental evidence suggest that these substances induce such behavioural responses by activating oxytocinergic neurons originating in the paraventricular nucleus and projecting to extrahypothalamic brain areas, i.e. the hippocampus, the ventral medulla and the spinal cord (see Argiolas and Melis, 1995, 1998). The molecular mechanisms for apomorphine, oxytocin and NMDA activation of oxytocinergic transmission to induce these behavioural responses, may involve Ca^{2+} and pertussis toxin-sensitive G proteins. First, the NMDA effect is prevented by MK-801, a potent non-competitive antagonist of the NMDA receptor (Wong et al., 1986), which blocks Ca^{2+} influx through the Ca^{2+} channel-coupled NMDA receptor. Second, either organic Ca^{2+} channel blockers or nanogram amounts of ω-conotoxin-GVIA, a potent blocker of N-type Ca^{2+} channels (McCleskey et al., 1987), prevent both apomorphine- and oxytocin-induced penile erection and yawning (Argiolas et al., 1989, 1990). Third, pertussis toxin, which inhibits the activity of several G proteins including the G_o protein

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coupled to voltage-dependent Ca^{2+} channels (see Dolphin, 1987), injected in the paraventricular nucleus prevents both apomorphine- and oxytocin-induced penile erection and yawning (Stancampiano et al., 1992).

We found that nitric oxide (NO) is involved at the paraventricular level in the control of penile erection and yawning induced by apomorphine, oxytocin and NMDA. First, the microinjection of N^G-nitro-L-arginine methyl ester, a potent and selective inhibitor of NO synthase (Rees et al., 1990), the Ca^{2+}-calmodulin-dependent enzyme that synthesizes NO from L-arginine (see Snyder, 1992; Moncada and Higgs, 1993; Southam and Garthwaite, 1993; Schuman and Madison, 1994) into the paraventricular nucleus inhibits the penile erection and yawning induced by the above substances (Melis et al., 1994a,b). Second, NO donors microinjected into the paraventricular nucleus induce penile erection and yawning indistinguishable from those induced by the above substances (Melis and Argiolas, 1995; Melis et al., 1995). Third, NO donor-induced penile erection and yawning are antagonized by the oxytocin receptor antagonist $[d(CH_2)_5Tyr(Me)^2\text{-}Orn^8]$vasotocin, given intracerebroventricularly (i.c.v.) (Melis and Argiolas, 1995; Melis et al., 1995). Since NO synthase is present in high concentrations in the paraventricular nucleus, including oxytocinergic cell bodies (Bredt et al., 1990; Vincent and Kimura, 1992; Sanchez et al., 1994; Amir, 1995), these findings led us to suggest that apomorphine, oxytocin and NMDA induce penile erection and yawning by increasing the Ca^{2+} influx that, in turn, activates NO synthase in the cell bodies of oxytocinergic neurons mediating these behavioural responses (Argiolas and Melis, 1995, 1998; Melis and Argiolas, 1997). The validity of this hypothesis was supported by results of in vivo microdialysis studies showing that dopamine receptor agonists, oxytocin and NMDA, when given at doses that induce penile erection and yawning, increase NO production in the paraventricular dialysate obtained from male rats (Melis et al., 1996, 1997a,b).

To further investigate the relationship between Ca^{2+} ions and NO in the control of penile erection and yawning induced by apomorphine, oxytocin and NMDA, we first used in vivo microdialysis in studies on the effect of ω-conotoxin injected into the paraventricular nucleus on the increase of paraventricular NO production induced by a dose of the above substances that induces penile erection and yawning. Second, we tested the effect of ω-conotoxin on penile erection and yawning induced by the NO donors, sodium nitroprusside and hydroxylamine.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (200–220 g) (Charles River, Como, Italy) were used in all the experiments. The 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 09:00–13:00 h.

2.2. Drugs and peptides

Apomorphine–HCl, sodium nitroprusside, hydroxylamine–HCl, sulfanilamide and *N*-(1-naphtyl)-ethylene-diamine were purchased from Sigma (St. Louis, MO, USA). NMDA (*N*-methyl-D-aspartic acid) and (5R,10S)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo [a,d]cyclo-hepten,5-10-imine hydrogen maleate ((+)-MK-801) from Research Biochemical International (Natick, MA, USA); oxytocin, $[d(CH_2)_5Tyr(Me)^2\text{-}Orn^8]$vasotocin and ω-conotoxin-GVIA, from Peninsula Eur. (St. Helens, UK), haloperidol from Janssen (Beerse, Belgium). All the other reagents were of the highest available purity.

2.3. Microinjections in the paraventricular nucleus and in vivo microdialysis

Stainless-steel guide cannulas (22 gauge, 0.71 mm) aimed unilaterally at the paraventricular nucleus were stereotaxically implanted (David Kopf Instruments, USA) under chloral hydrate anaesthesia two days before the experiments (coordinates: 0.2 mm anterior to bregma, 0.4 mm lateral to midline and 2.0 mm ventral to dura) (Pellegrino and Cushman, 1971). Each rat was used only once. For paraventricular injections, substances dissolved in saline or saline alone (control rats), were injected in a volume of 0.3 μl in 2 min via an internal cannula that extended 5.3 mm below the tip of the guide cannula to reach the paraventricular nucleus (see Pellegrino and Cushman, 1971). The internal cannula was connected by polypropylene tubing to a 10-μl Hamilton syringe driven by a Stoelting microinfusion pump as already described (Melis et al., 1995; Melis and Argiolas, 1995). After microinjection, the tip of the cannula was left in the injection site for 30 s to allow spreading of the injected solution.

For microinjections and microdialysis in the paraventricular nucleus of the same animal, microdialysis probes with approximately 1 mm of free surface for dialysis, in a loop flow design, and with an infusion cannula made from fused capillary silica tubing ending adjacent to the U-shaped membrane glued to the microdialysis probe with epoxy resin were prepared as already described (Melis et al., 1997b). The probes were then implanted stereotaxically in the paraventricular nucleus as described above, under chloral hydrate anaesthesia, two days before the experiments. Each rat was used only once. During the experiments, the probes were perfused with a 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 the Stoelting 200 microsyringe pump.

2.4. Behavioural studies

During microdialysis, the rats implanted with the modified microdialysis probe 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 at one end and to the polyethylene collecting loop at the other end. The cannula for paraventricular injections was also connected to a 10-μl Hamilton microsyringe driven by a microsyringe pump via polyethylene tubing. After a 2-h equilibration period with perfusion of the dialysis probe with Ringer's solution at a constant flow rate of 2 μl/min, the dialysate was collected every 20 min (in fractions of 40 μl) in polyethylene tubing loops and transferred into polyethylene tubes at 10–15°C for the determination of NO_2^- and NO_3^- as described below. Apomorphine, oxytocin or NMDA 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 ω-conotoxin was injected in the paraventricular nucleus over a period of 2 min, 15 min before apomorphine, oxytocin or NMDA. After treatments, the 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.

In experiments in which microdialysis was not performed, saline, ω-conotoxin, [d$(CH_2)_5$Tyr$(Me)^2$-Orn^8]vasotocin or MK-801 was given into the paraventricular nucleus of rats implanted with chronic guide cannulas over a 2-min period, 15 min before saline, sodium nitroprusside or hydroxylamine. The nitric oxide donors were protected from light with aluminium foil wrapped around the syringe and polypropylene tubing. When haloperidol was used, the drug was dissolved in a drop of concentrated acetic acid, diluted with saline (final pH = 5.0–5.3) and injected intraperitonaelly (i.p.) in a volume of 1 ml/rat, 15 min before sodium nitroprusside or hydroxylamine. Shortly after treatment, the animals were placed individually into Plexiglas cages (30 × 30 × 30 cm) and observed for 60 min, during which penile erection and yawning episodes were counted by an observer who was not aware of the treatments to eliminate subjective evaluations.

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-naphtyl)-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). 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 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, as already described (Melis et al., 1996). Total NO_2^- was then determined as described above and the amount of NO_3^- was calculated by subtracting 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. 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 injection site and/or the position of the tip of the dialysis probe, 50-μm transverse brain sections were prepared by means of a freezing microtome, were stained with Neutral Red and inspected on a phase-contrast microscope. The injection site and/or the position of the probe tip was localized by following the cannula and/or the probe tract through a series of brain sections. Only those animals found to have the probe or the internal cannula tip positioned correctly in the paraventricular nucleus were included for the statistical evaluation of the results.

2.7. Statistics

Statistical evaluation of the results was performed by two-way analysis of variance (ANOVA) for repeated measures, followed by Duncan's multiple range test when NO_2^-, NO_3^-, penile erection and yawning were measured in the same experiment. One-way ANOVA followed by Duncan's multiple range test was used to evaluate the results of those experiments in which penile erection and yawning only were quantified. In both cases a $P < 0.05$ was considered significant (Tallarida and Murray, 1986).

3. Results

3.1. Effect of ω-conotoxin on the apomorphine-induced increase of NO_2^- and NO_3^- concentration, penile erection and yawning

Apomorphine (50 ng), given into the paraventricular nucleus after a 2-h equilibration period, increased the concentration of NO_2^- from 0.98 ± 0.20 μM to 3.8 ± 0.65 μM and that of NO_3^- from 5.51 ± 0.81 to 10.9 ± 1.75 μM in the paraventricular dialysate. The increase was already evident 20 min after treatment, reached its maximum at 40

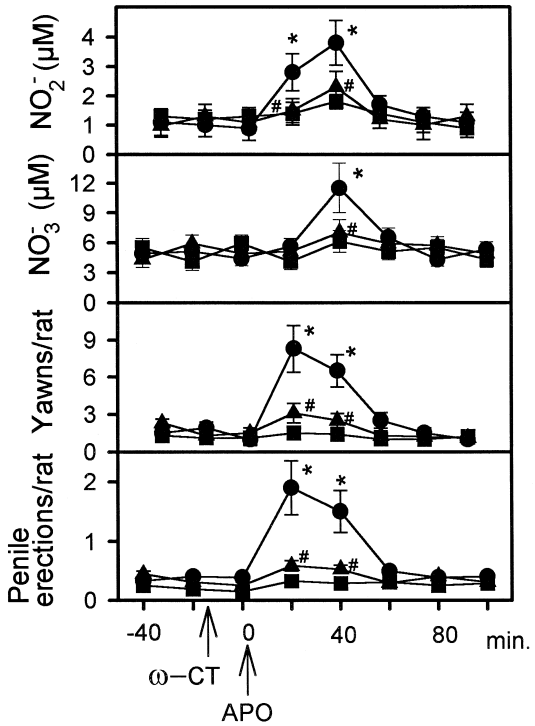

Fig. 1. Effect of ω-conotoxin on the apomorphine-induced increase in paraventricular NO_2^-/NO_3^- concentration, penile erection and yawning. Rats were placed individually into a Plexiglas cage and perfused with Ringer solution as described in Section 2. After a 2-h period of perfusion with Ringer's solution, Ringer's solution (0.3 μl) or ω-conotoxin (5 ng) was injected in the paraventricular nucleus 15 min before apomorphine (50 ng). Filled circles represent Ringer's solution + apomorphine-treated rats, filled squares, ω-conotoxin + Ringer's solution-treated rats, filled triangles, ω-conotoxin + apomorphine-treated rats. 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. for 7 rats. $*P < 0.01$ with respect to pretreatment values (negative times); $\#P < 0.01$ with respect to the corresponding Ringer's solution + apomorphine-treated rats (Two-way ANOVA followed by Duncan's multiple range test).

min and disappeared 60 min later (Fig. 1). As expected, the NO_2^- and NO_3^- increase was concomitant with a significant increase in the number of penile erection and yawning episodes. The apomorphine-induced NO_2^- and NO_3^- increase, penile erection and yawning were prevented by ω-conotoxin (5 ng), given into the paraventricular nucleus 15 min before the dopamine receptor agonist. The toxin given alone slightly increased NO_2^- (from 1.15 ± 0.3 μM to 1.85 ± 0.32 μM), was ineffective to induce penile erection and yawning and it did not induce any gross behavioural change. Two-way ANOVA for repeated measures revealed a significant effect of treatment (ω-conotoxin) ($F(1,80) = 117.69$, 188.48, 29.01 and 5.65, $P < 0.01$) and time ($F(7,80) = 69.69$, 167.34, 58.70 and 9.22, $P < 0.01$) on apomorphine-induced penile erection, yawning and NO_2^- and NO_3^- concentration, as well as a treatment × time interaction ($F(7,80) = 39.77$, 75.05, 11.66 and 4.68, $P < 0.01$).

3.2. Effect of ω-conotoxin on the oxytocin-induced increase of NO_2^- and NO_3^- concentration, penile erection and yawning

Like apomorphine, oxytocin (30 ng) given into the paraventricular nucleus increased the concentration of NO_2^- from 1.15 ± 0.32 μM to 4.2 ± 0.63 μM and that of NO_3^- from 4.89 ± 0.79 to 10.5 ± 1.98 μM in the paraventricular dialysate. The increase was already evident 20 min after the injection, reached its maximum at 40 min, and the values returned to their basal levels 80 min later (Fig. 2). As expected, the NO_2^- and NO_3 increase was concomitant with penile erection and yawning (Fig. 2). As found with apomorphine, the oxytocin-induced increase in NO_2^- and NO_3^- concentration, penile erection and yawning were not observed when ω-conotoxin (5 ng) was injected in the paraventricular nucleus 15 min before oxytocin. Two-way

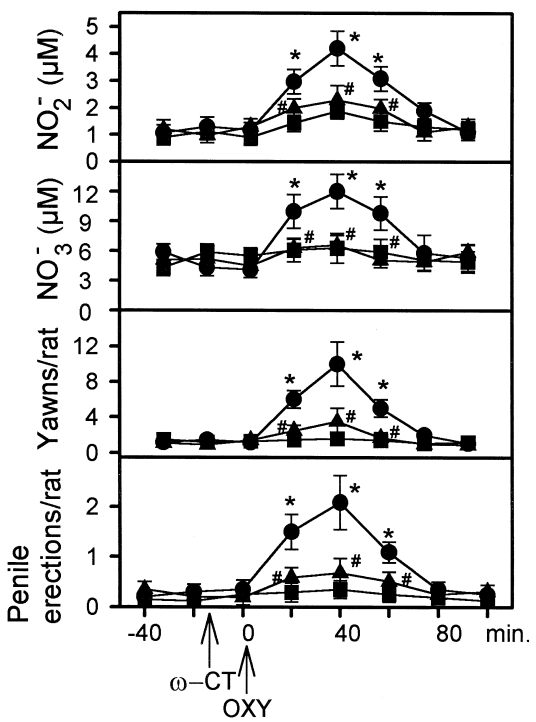

Fig. 2. Effect of ω-conotoxin on the oxytocin-induced increase in paraventricular NO_2^-/NO_3^- concentration, penile erection and yawning. Experimental conditions were identical to those described in the legend of Fig. 1, except that oxytocin (30 ng) was used instead of apomorphine. Filled circles represent Ringer's solution + oxytocin-treated rats, filled squares ω-conotoxin + Ringer's solution-treated rats, filled triangles ω-conotoxin + oxytocin-treated rats. The dialysate was analyzed for its 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. for 7 rats. $*P < 0.01$ with respect to pretreatment values (negative times); $\#P < 0.01$ with respect to the corresponding Ringer's solution + oxytocin-treated rats (two-way ANOVA followed by Duncan's multiple range test).

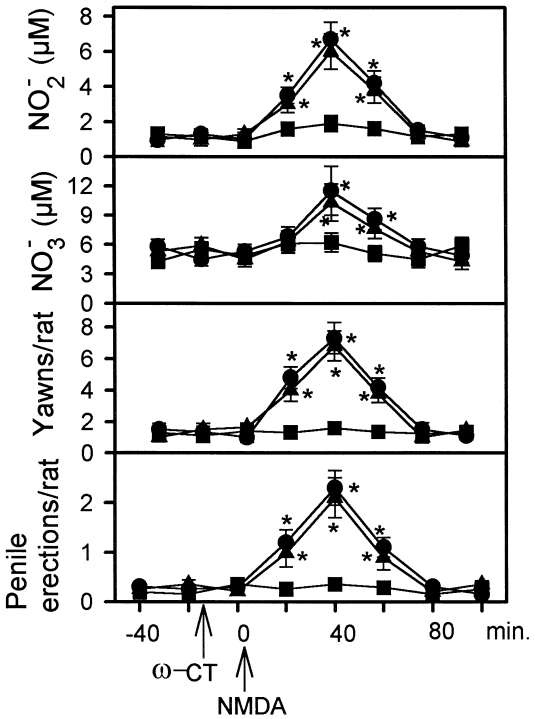

Fig. 3. Effect of ω-conotoxin on the NMDA-induced increase in paraventricular NO_2^-/NO_3^- concentration, penile erection and yawning. Experimental conditions were identical to those described in the legend of Fig. 1, except that NMDA (50 ng) was used instead of apomorphine. Filled circles represent Ringer's solution + NMDA-treated rats, filled squares, ω-conotoxin + Ringer's solution-treated rats, filled triangles, ω-conotoxin + NMDA-treated rats. The dialysate was analyzed for its 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. for six rats. $* P < 0.01$ with respect to pretreatment values (negative times) (two-way ANOVA followed by Duncan's multiple range test).

ANOVA revealed a significant effect of treatment (ω-conotoxin) ($F(1,80) = 162.20$, 362.57, 41.58 and 18.17, $P < 0.01$) and time ($F(7,80) = 85.18$, 222.43, 55.23 and 7.71, $P < 0.01$) on oxytocin-induced penile erection, yawning and NO_2^- and NO_3^- concentration, as well as a drug × time interaction ($F(7,80) = 45.43$, 80.34, 16.24 and 7.71, $P < 0.01$).

3.3. Effect of ω-conotoxin on the NMDA-induced increase of NO_2^- and NO_3^- concentration, penile erection and yawning

Like apomorphine and oxytocin, NMDA (50 ng) given into the paraventricular nucleus increased the concentration of NO_2^- from 1.25 ± 0.29 μM to 6.7 ± 0.98 μM and that of NO_3^- from 5.01 ± 0.75 to 11.2 ± 1.85 μM in the paraventricular dialysate. The increase was already evident 20 min after the injection, reached its maximum at 40 min and disappeared 80 min later (Fig. 3). The NMDA-induced NO_2^- and NO_3^- increase was also associated with the appearance of penile erection and yawning (Fig. 3). In contrast to apomorphine and oxytocin responses, the NMDA-induced NO_2^- and NO_3^- increase, penile erection and yawning were not prevented by ω-conotoxin (5 ng) injected into the paraventricular nucleus 15 min before NMDA. Two-way ANOVA failed to reveal a significant effect of ω-conotoxin ($F(1,80) = 2.30$, 2.24, 0.17 and 1.06, $P > 0.1$, not significant) on NMDA-induced penile erection, yawning, NO_2^- and NO_3^- concentration.

3.4. Effect of ω-conotoxin on nitric oxide donor-induced penile erection and yawning: comparison with effects of haloperidol, [d(CH$_2$)$_5$Tyr(Me)^2-Orn^8]vasotocin and MK-801

Sodium nitroprusside and hydroxylamine, each given at the dose of 50 μg into the paraventricular nucleus, induced penile erection and yawning episodes indistinguishable from those induced by apomorphine, oxytocin or NMDA (Table 1). MK-801 (50 ng), [d(CH$_2$)$_5$Tyr(Me)^2-Orn^8]vasotocin (100 ng) and ω-conotoxin (5 ng) injected into the paraventricular nucleus 15 min before sodium nitroprusside or hydroxylamine, and haloperidol (0.5 mg/kg i.p.) given 30 min before the NO donors, were all unable to prevent penile erection and yawning induced by the NO donors (Table 1). One-way ANOVA failed to

Table 1
NO donor-induced penile erection and yawning: effect of ω-conotoxin, haloperidol, MK-801 and [d(CH$_2$)$_5$Tyr(Me)^2-Orn^8]-vasotocin

Pretreatment	Treatment					
	Saline		Sodium nitroprusside		Hydroxylamine	
	Penile erections/rat	Yawns/rat	Penile erections/rat	Yawns/rat	Penile erections/rat	Yawns/rat
Saline (0.3 μl)	0.5 ± 0.2	2.0 ± 0.4	2.9 ± 0.48[a]	15.9 ± 2.9[a]	3.2 ± 0.49[a]	18.2 ± 3.5[a]
ω-Conotoxin (5 ng)	0.3 ± 0.1	1.5 ± 0.3	3.1 ± 0.45[a]	16.5 ± 3.5[a]	2.8 ± 0.40[a]	16.3 ± 3.2[a]
[d(CH$_2$)$_5$Tyr(Me)^2-Orn^8]-VT (0.1 μg)	0.4 ± 0.2	1.4 ± 0.5	2.3 ± 0.39[a]	16.1 ± 2.5[a]	2.5 ± 0.49[a]	18.3 ± 3.5[a]
Haloperidol (0.5 mg/kg i.p.)	0.5 ± 0.1	1.2 ± 0.4	2.5 ± 0.32[a]	17.3 ± 3.1[a]	3.1 ± 0.48[a]	17.8 ± 3.4[a]
MK-801 (50 ng)	0.3 ± 0.2	1.8 ± 0.5	3.0 ± 0.45[a]	17.6 ± 2.5[a]	2.8 ± 0.31[a]	18.5 ± 3.5[a]

Sodium nitroprusside or hydroxylamine was dissolved in saline and injected into the paraventricular nucleus in a volume of 0.3 μl. MK-801, ω-conotoxin, or [d(CH$_2$)$_5$ Tyr(Me)^2-Orn^8]vasotocin was injected into the paraventricular nucleus in a volume of 0.3 μl of saline 15 min before sodium nitroprusside or hydroxylamine, while haloperidol was given i.p. 30 min before either NO donor. After treatment, the rats were placed individually into a Plexiglas cage and observed for 60 min, during which penile erection and yawning episodes were counted. Each value is the mean ± S.E.M. for eight rats.
[a] $P < 0.01$ with respect to the corresponding group of saline-treated rats.

reveal any effect of ω-conotoxin, haloperidol, [d$(CH_2)_5$-Tyr(Me)^2-Orn^8]vasotocin or MK-801 on NO donor-induced penile erection and yawning.

4. Discussion

The present results showed that ω-conotoxin, a potent and selective blocker of N-type Ca^{2+} channels (McCleskey et al., 1987), injected into the paraventricular nucleus prevents the apomorphine- and oxytocin-induced increase of NO_2^- and NO_3^- concentration in the paraventricular dialysate, and the penile erection and yawning. This agrees with the results of previous studies showing that penile erection and yawning induced by apomorphine or oxytocin were prevented by ω-conotoxin given either i.c.v. or directly into the paraventricular nucleus (Argiolas et al., 1990). Since NO_2^- and NO_3^- are the products of the reaction of newly synthetized NO with O_2 (Ignarro, 1990; Luo et al., 1993; Ohta et al., 1994; Melis et al., 1996), and NO is synthesized by the Ca^{2+}-calmodulin-dependent NO synthase (Snyder, 1992; Moncada and Higgs, 1993; Southam and Garthwaite, 1993; Schuman and Madison, 1994) these results provide further support for the hypothesis that apomorphine and oxytocin increase the Ca^{2+} concentration in the cell bodies of paraventricular oxytocinergic neurons mediating penile erection and yawning. The increased Ca^{2+} concentration, in turn, activates NO synthase in these oxytocinergic neurons (see Section 1). Most important, the potency of ω-conotoxin to prevent the increase in NO production, penile erection and yawning induced by apomorphine or by oxytocin confirms that Ca^{2+} influx through N-type Ca^{2+} channels plays an important role in the activation of NO synthase and consequent behavioural responses induced by these compounds, as previously suggested (Melis et al., 1997a,b). As to the mechanism by means of which the activation of dopamine or oxytocin receptors may cause the opening of ω-conotoxin-sensitive Ca^{2+} channels, one possibility is that both dopamine and oxytocin receptors in the paraventricular nucleus are coupled through a G protein, directly to ω-conotoxin-sensitive Ca^{2+} channels or to a still unidentified transduction system, which leads to the opening of ω-conotoxin-sensitive Ca^{2+} channels. Were the latter hypothesis correct, opening of ω-conotoxin-sensitive Ca^{2+} channels would be mediated by changes in the content of second messengers (e.g. diacylglycerol or inosytol-triphospate) as found in other tissues (for a review on the transduction systems coupled to dopamine receptors see Baldessarini, 1996 and for those coupled to oxytocin receptors see Argiolas and Gessa, 1991 and Lambert et al., 1994). In line with this possibility, pertussis toxin, which inhibits several G proteins (for a review see Dolphin, 1987), injected into the paraventricular nucleus prevents both apomorphine- and oxytocin-induced penile erection and yawning (Stancampiano et al., 1992).

The above explanation is based mainly on the assumption that both apomorphine and oxytocin act directly on receptors located in the cell bodies of oxytocinergic neurons mediating penile erection and yawning and that ω-conotoxin-sensitive Ca^{2+} channels are located in the same neurons. However ω-conotoxin might prevent apomorphine and oxytocin responses by inhibiting ω-conotoxin-sensitive Ca^{2+} channels located presynaptically, that is by inhibiting the release of other neurotransmitters and/or neuropeptides that activate oxytocinergic neurons to induce penile erection and yawning, i.e. dopamine, excitatory amino acids or oxytocin itself. Were this the case, apomorphine would increase the release of oxytocin or excitatory amino acids, to induce the above responses, while oxytocin would increase the release of dopamine or excitatory amino acids. However, several findings argue against this possibility. First, MK-801, which blocks NMDA receptors (Wong et al., 1986), injected into the paraventricular nucleus is unable to prevent penile erection and yawning induced by apomorphine or by oxytocin, despite preventing NMDA-induced penile erection, yawning and the concomitant increase in paraventricular NO production (Melis et al., 1997b). Second, haloperidol, which blocks dopamine receptors, is unable to prevent penile erection and yawning induced by oxytocin or by NMDA, despite its ability to prevent apomorphine-induced penile erection, yawning and the concomitant increase in paraventricular NO production (Melis et al., 1996, 1997a,b). Third, [d$(CH_2)_5$Tyr(Me)^2-Orn^8]vasotocin, which blocks oxytocinergic receptors (Bankowski et al., 1980), is unable to prevent apomorphine- and NMDA-induced penile erection, yawning and the concomitant increase in paraventricular NO production, when injected into the paraventricular nucleus (Melis et al., 1996, 1997b), despite its ability to prevent oxytocin responses (Melis et al., 1997a).

In contrast, ω-conotoxin does not prevent NMDA-induced NO production, penile erection and yawning, while it prevents these responses when they are induced by apomorphine and oxytocin. Since these NMDA responses are easily explained if NMDA increases the Ca^{2+} concentration in the cell bodies of oxytocinergic neurons mediating penile erection and yawning by opening NMDA receptor-coupled Ca^{2+} channels (see Melis et al., 1997b), the finding suggests that ω-conotoxin-sensitive Ca^{2+} channels play only a minor role, if any, in the NMDA-induced activation of NO synthase of paraventricular oxytocinergic neurons mediating the above behavioural responses. A similar failure of ω-conotoxin to prevent NMDA-induced NO production was found, for instance, in cultured striatal neurons (Rodriguez-Alvarez et al., 1997). The inability of ω-conotoxin to prevent NO synthase activation by NMDA is in line with the hypothesis that NMDA does not act by modulating the release of other neurotransmitters and/or

neuropeptides present in the paraventricular nucleus, such as excitatory amino acids (Van den Pol, 1991), dopamine (Buijs et al., 1984; Lindvall et al., 1984) or oxytocin itself (see Argiolas and Gessa, 1991).

The present results also showed that ω-conotoxin does not prevent penile erection and yawning induced by the NO donors sodium nitroprusside and hydroxylamine when they are injected into the paraventricular nucleus. The finding is in line with the hypothesis that NO formed by these compounds (and endogenous NO as well) acts as an intracellular messenger inside the oxytocinergic neurons mediating penile erection and yawning (see Argiolas and Melis, 1995, 1998; Melis and Argiolas, 1997), rather than by modulating Ca^{2+} influx through ω-conotoxin-sensitive Ca^{2+} channels. Interestingly, NO donors modulate NMDA receptor-coupled Ca^{2+} channels (Kiedrowski et al., 1992; Hoyt et al., 1992) and other ionic channels as well, through cyclic guanosine 3,5′monophosphate (c-GMP)-dependent and -independent mechanisms in brain tissues and neuronal cell lines (Chen and Schofiels, 1995; Clementi et al., 1995; Koh et al., 1995; Sawada et al., 1995). However, since MK-801, which blocks NMDA receptor-coupled Ca^{2+} channels (Wong et al., 1986), is unable to prevent NO donor-induced penile erection and yawning, it is unlikely that NO donors modulate NMDA receptors in the paraventricular nucleus to induce these behavioural responses. Conversely, the inability of MK-801 to prevent NO donor-induced penile erection and yawning suggests that these compounds do not release excitatory amino acids in the paraventricular nucleus to induce these behavioural responses. Since NO donor-induced penile erection and yawning are prevented by neither oxytocin receptors antagonists given into the paraventricular nucleus (Melis and Argiolas, 1995; Melis et al., 1995) nor haloperidol, which blocks dopamine receptors (Melis et al., 1995 and this study), it is unlikely that NO donors modulate apomorphine or oxytocin receptors or facilitate the release of endogenous dopamine or oxytocin in the paraventricular nucleus to induce these behavioural responses.

Unfortunately the present results do not clarify the mechanism(s) by means of which endogenous or NO donor-derived NO activates oxytocinergic transmission at the paraventricular level to induce penile erection and yawning. c-GMP is apparently not involved, at least at the paraventricular level, in these behavioural responses, although a role of guanylate cyclase in sites distant from the paraventricular nucleus cannot be ruled out (Melis et al., 1994a,b, 1995, 1996, 1997a,b; Melis and Argiolas, 1995). Since Ca^{2+} influx plays a key role in the increase of NO production, penile erection and yawning induced by apomorphine, NMDA and oxytocin, it is tempting to speculate that NO donors induce changes in intracellular Ca^{2+} similar to those induced by the above compounds in the paraventricular oxytocinergic neurons mediating these behavioral responses. It is consistent with this possibility that NO donors induce complex changes in intracellular Ca^{2+} in different cell lines and brain tissues (Kiedrowski et al., 1992; Hoyt et al., 1992; Clementi et al., 1995; Volk et al., 1997).

In conclusion the present results are compatible with the hypothesis that apomorphine, oxytocin and NMDA increase NO synthesis directly in the oxytocinergic neurons mediating penile erection and yawning, rather than by modulating the release of other neurotransmitters and/or neuropeptides in the paraventricular nucleus. However, while ω-conotoxin-sensitive Ca^{2+} channels play an important role when the above responses are induced by apomorphine or by oxytocin, these Ca^{2+} channels seem to play only a minor role, if any, when the responses are induced by NMDA or by NO donors.

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