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Nitric oxide is involved in male sexual behavior of rats

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Abstract

In male rats, whether sexually experienced or sexually naive, the intraperitoneal administration of L-arginine (the natural substrate for nitric oxide synthase) (10, 25, 50 mg/kg) both increased the percentage of copulating in sexually naive rats and improved the indexes of sexual performance in sexually experienced rats, whereas the intraperitoneal administration of N^G-nitro-L-arginine methyl ester (L-NAME) (a potent inhibitor of nitric oxide synthase) (10, 25, 50 mg/kg) had opposite effects. In contrast, after intracerebroventricular administration, L-arginine (25, 50, 100 μg/rat) had no effect – whether in naive or in experienced rats – whereas L-NAME completely prevented ejaculation in naive rats, at the dose of 100 μg/rat, but had no effect at all in experienced rats, up to the dose of 300 μg/rat. Finally, a direct relationship seems to exist between male copulatory performance and nitric oxide synthase activity in a discrete and defined brain area, the paraventricular nucleus of the hypothalamus: indeed, nitric oxide synthase mRNA expression in this nucleus in sexually potent rats is about twice that in sexually impotent rats. It is concluded that nitric oxide synthase is involved in the expression of male sexual activity, in spite of some inconsistencies that are hard to interpret.

Keywords: Sexual behavior; Nitric oxide (NO); Nitric oxide (NO) synthase; Paraventricular nucleus; (Rat)

1. Introduction

Nitric oxide (NO) is an atypical regulatory molecule that acts both as a second messenger and as a neurotransmitter, and has been implicated in diverse physiological functions, ranging from bactericidal and tumoricidal actions of macrophages to blood vessel relaxation (for a review see: Moncada et al., 1991). Accumulating evidence indicates that NO may also be a major neuronal messenger (Snyder, 1992). In particular, it is an established physiological mediator of penile erection (Förstermann et al., 1990) and, in the brain, NO synthase is highly concentrated in structures directly or indirectly involved in sexual behavior (olfactory bulb, supraoptic and paraventricular nuclei, amygdala, septal structures, etc.) (Bredt et al., 1991). This may suggest a more comprehensive role for NO in copulation, not limited to the induction of penile erection by an action in the periphery, but also including the behavioral component of sexual activity.

Our present research was accordingly aimed at investigating (i) the effect of NO synthesis stimulation or inhibition on male sexual behavior, and (ii) the possible existence of differences in NO synthase expression in discrete brain areas implicated in sexual behavior in sexually potent and sexually impotent male rats.

2. Materials and methods

2.1. Animals and surgery

Adult male and female Wistar rats were obtained from Morini (S. Polo d'Enza, Reggio nell'Emilia, Italy) and maintained three per Plexiglas cage (25 × 40 × 15 cm), males and females separately, in temperature-controlled colony rooms (22 ± 1°C; 60% humidity) on a 12 h light/dark cycle, with lights off at 7 a.m. The rats were 3-months-old at the beginning of the experiment, and weighed 240–270 g (males) and 180–210 g

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(females). Food and water were available ad libitum. The males to be intracerebroventricularly (i.c.v.) treated were implanted, under ketamine plus xylazine anesthesia (120 + 2 mg/kg i.p.) (Farmaceutici Gellini, Aprilia, Italy; Bayer, Milano, Italy), with permanent stainless-steel guide cannulae (Plastic Products Co., Roanoke, VA, USA) aimed at a brain lateral ventricle (Paxinos and Watson, 1982) and fixed to the skull with screws and dental acrylic cement. Animals so prepared were thereafter kept in individual cages and were used for the experiments at least 5 days after surgery. Correct guide cannula placement was verified at the end of the experiment by injecting 4 μl of toluidine blue dye, followed by decapitation under ethyl ether anesthesia and dissection of the brain.

The females were ovariectomized under ethyl ether anesthesia and brought into estrus by the subcutaneous (s.c.) injection of 15 μg estradiol benzoate followed, 48 h later, by the s.c. injection of 500 μg of progesterone. They were screened with non-experimental sexually experienced males and only those exhibiting good sexual receptivity (solicitation behavior and lordosis in response to mounting) and no rejection behavior, were used.

2.2. Behavioral testing

Copulatory behavior tests were performed during the period of darkness (between 10:00 and 14:00 h) in a soundproof room, under a dim red light, according to the standard procedure (Dewsbury, 1972; Clark et al., 1987). After a 10-min adaptation period in a rectangular glass observation cage (60 × 50 × 40 cm), a stimulus female was presented to the male by dropping it gently into the cage. The following behavioral parameters were recorded or calculated: mount latency and intromission latency, the time from introduction of the female to the occurrence of the first mount or intromission; ejaculation latency, the time from the first intromission to ejaculation; post-ejaculatory interval, the time from ejaculation to the subsequent intromission; mount frequency and intromission frequency, the number of mounts and intromissions preceding ejaculation; intercopulatory interval, the average interval between successive intromissions (calculated as ejaculation latency divided by intromission frequency); copulatory efficacy, a measure of intromissive success (calculated as intromission frequency divided by mount frequency + intromission frequency).

Mount latency, intromission latency, ejaculation latency, post-ejaculatory interval and intercopulatory interval are considered to be inversely proportional to arousal/motivation, while intromission frequency and between intromissions interval are considered to be indicative of performance/potency (Beach, 1956; Clark et al., 1987). Tests were terminated if intromission did not occur within 15 min of female introduction, if ejaculation latency exceeded 30 min, post-ejaculatory interval exceeded 15 min, or immediately after the post-ejaculatory intromission.

Rats were used for the study either without previous sexual training (sexually naive) or after seven training tests with sexually receptive females, at 5- or 6-day intervals; in this latter case, only those males (80%) satisfying the above described criteria of sexual vigor in at least the last three preliminary, pre-experimental tests (sexually experienced and potent) were used. On the other hand, males failing to achieve ejaculation in all the last three pre-experimental tests (impotent rats) were used only for the in situ hybridization study.

2.3. In situ hybridization study

Six sexually potent and six impotent male rats were decapitated. The brains were rapidly removed and frozen on dry ice. 20 μm thick sections through the paraventricular nucleus of the hypothalamus, the bed nucleus of the stria terminalis, and the amygdala were cut in a cryostat (Leitz, Wetzlar, Germany) and thaw-mounted onto precleaned microscope glass slides (Probe On, Fisher Scientific, Pittsburgh, PA, USA). An oligonucleotide probe with sequence complementary to mRNA for rat NO synthase (nucleotides 795–840) of the cloned DNA (Bredt et al., 1991) was synthetized using the Beckmann Oligo 1000 synthesizer. The oligonucleotide was then labelled at the 3′-end using terminal deoxynucleotidyl transferase (IBI, New Haven City, CT, USA) in a cobalt-containing buffer and α-^{35}S-dATP (New England Nuclear, Boston, MA, USA) to a specific activity of $1–3 \times 10^6$ dpm ng^{-1} and purified on a Nensorb-20 column (New England Nuclear). Sections were hybridized, without prior fixation, for 18 h at 42°C in a humidified box with 10^6 dpm 100 μl^{-1} of probe in a mixture of 4 × SSC (saline sodium citrate) (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate), 50% formamide, 1 × Denhardt's solution (0.02% each of polyvinyl-pyrrolidone, bovine serum albumin and Ficoll), 1% sarcosyl, 0.02 M phosphate buffer (pH 7.0), 10% dextran sulphate, 250 μg ml^{-1} yeast tRNA, 500 μg ml^{-1} heat-denatured salmon sperm DNA and 200 mM dithiothreitol. Following hybridization, the sections were rinsed in 1 × SSC (4 times for 15 min each) at 55°C followed by a 1-h wash at room temperature, dipped in distilled water, transferred through 60% and 90% ethanol and air-dried. Afterwards the slides were placed in a cassette, covered with Hyperfilm β-max X-ray film (Amersham) and exposed at −20°C for 7–20 days according to the brain area. The films were developed using D19 developer (Kodak) and Hypam fixative (Ilford, Cheshire, England). Densitometrical analysis of the film was performed using the Leica Quantimet 520 + image analyser. The nuclei were

identified after toluidine staining of the sections. Using an adjustable window in order to sample the labelled areas of the nuclei, four rostro-caudal matching sections of the nuclei were measured and the mean ± S.E. was first calculated for each animal. The mean grey value measured in each sampled area was converted into radioactivity value using an appropriate standard curve.

2.4. Drugs and treatments

L-Arginine and N^G-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). They were dissolved in saline and diluted to the appropriate concentrations immediately before use. Intraperitoneal (i.p.) injections were in a volume of 0.1 ml per 100 g b.w.; i.c.v. injections were in a volume of 5 μl/rat, at the rate of 1 μl/20 s, via the i.c.v. internal cannula connected by polyethylene tubing to a 10 μl Hamilton syringe driven by a micrometric screw. I.p. doses were: L-arginine, 10, 25, 50 mg/kg 15 min before testing; L-NAME, 10, 25, 50 mg/kg 60 min before testing. The following doses were used for the i.c.v. route: L-arginine, 25, 50, 100 μg/rat; L-NAME, 25, 50, 100 and 300 μg/rat immediately before testing. Each rat received only one treatment. Each group comprised 10–13 rats.

2.5. Data analysis

The data are presented as means ± S.E.M.; the behavioral data were analyzed using a Kruskall-Wallis analysis of variance (ANOVA) followed by Mann-Whitney U-test and Fisher's exact test, and the biochemical data using Student's *t*-test. A P level of < 0.05 was considered significant.

3. Results

3.1. Copulatory behavior of sexually naive rats

The i.p. administration of L-arginine (10, 25 or 50 mg/kg), 15 min before testing, significantly increased the percentage of animals which mounted the receptive female as well as the percentage of animals which achieved ejaculation (Fig. 1). Conversely, the percentage of mounting and ejaculating animals, which is already low in these sexually naive rats, was further reduced by the i.p. administration of L-NAME, albeit only at the highest dose used (50 mg/kg) (Fig. 1). On the other hand, the i.c.v. administration of L-arginine (25, 50 or 100 μg/rat), immediately before testing, had no influence on the percentage of either mounting or ejaculating animals, while the i.c.v. administration of L-NAME reduced the percentage of mounting rats and completely prevented ejaculation at the dose of 100 μg/rat. This effect was not increased by further increasing the dose (300 μg/rat) (data not shown).

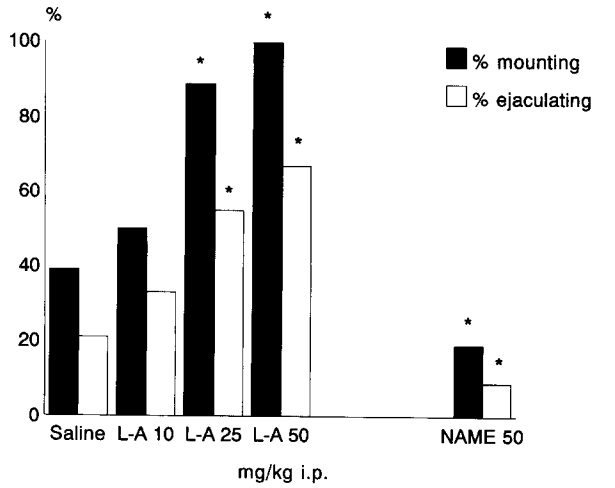

Fig. 1. Percentage of mounting and ejaculating male rats. Rats were sexually naive. L-Arginine (L-A) and N^G-nitro-L-arginine methyl ester (NAME) were intraperitoneally (i.p.) administered 15 min and 60 min, respectively, before test (10–13 rats per group). * $P < 0.05$, at least, compared with saline-treated controls (Fisher's test).

3.2. Copulatory behavior of sexually experienced rats

The i.p. administration of L-arginine further improved the already vigorous copulatory activity of these animals, the maximum effect being obtained with the dose of 25 mg/kg (Table 1). Obviously, the percentage of mounting and ejaculating animals could not be fur-

Table 1
Influence of intraperitoneal administration of L-arginine (L-A) on male sexual behavior, in sexually experienced rats

Treatment (mg/kg)	ML (s)	IL (s)	EL (s)	MF	IF (No.)	PEI (No.)	ICI (s)	CE (s)
Saline	103.6 ± 70.4	219.2 ± 92.5	741.0 ± 137.4	4.4 ±0.8	14.0 ± 1.9	468.7 ± 59.7	52.9	0.76
L-A, 10	83.5 ± 48.2	181.4 ± 87.0	594.3 ± 96.1	3.8 ±0.6	11.7 ± 1.8	513.2 ± 71.2	50.8	0.75
L-A, 25	23.3 ± 12.7 [a]	45.6 ± 18.7 [a]	380.8 ± 38.9 [a]	3.2 ±0.4	13.0 ± 2.4	378.6 ± 36.9	29.9	0.80
L-A, 50	94.5 ± 32.8	168.3 ± 78.2	466.6 ± 70.7	2.70 ± 0.2	13.3 ± 2.1	387.1 ± 22.1	35.0	0.83

Groups of 10–13 animals. Treatment was given 15 min prior to the test. Each value is the mean ± S.E.M. [a] $P < 0.05$ compared with saline-treated controls (Mann-Whitney U-test). ML = mount latency; IL = intromission latency; EL = ejaculation latency; MF = mount frequency; IF = intromission frequency; PEI = post-ejaculatory interval; ICI = intercopulatory interval; CE = copulatory efficacy.

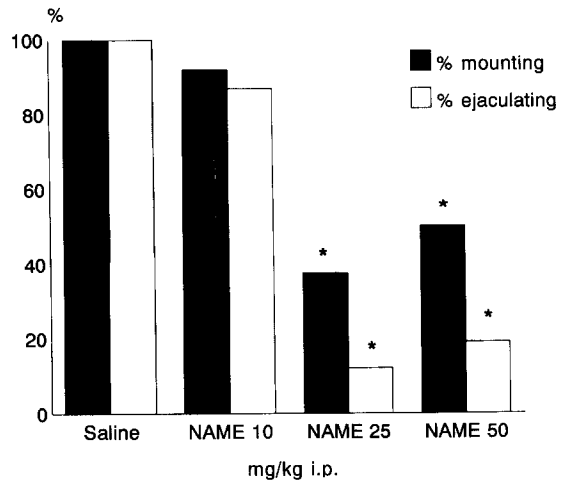

Fig. 2. Percentage of mounting and ejaculating male rats. Rats were sexually experienced. N^G-nitro-L-arginine methyl ester (NAME) was intraperitoneally (i.p.) administered 60 min before test (10–13 rats per group). * $P < 0.05$, at least, compared with saline-treated controls (Fisher's test).

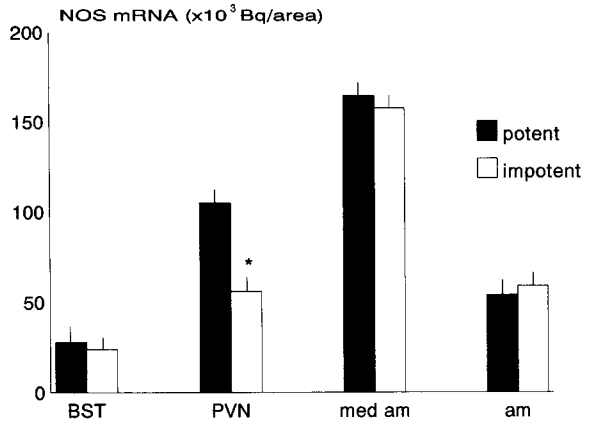

Fig. 3. Localization of NO synthase mRNA in brain of sexually potent and impotent male rats. Among the areas examined (bed nucleus of stria terminalis = BST; parvocellular paraventricular nucleus of hypotalamus = pPVN; magnocellular paraventricular nucleus of the hypothalamus = mPVN; median amygdala = med am; amygdala in toto = am), the difference in radioactivity is significant only for the paraventricular nucleus of the hypothalamus. * $P < 0.05$, (Student's t-test).

ther improved, already being 100%. On the other hand, the administration of L-NAME by this same route significantly reduced the percentage of mounting and ejaculating animals – the maximum inhibition being obtained with the dose of 25 mg/kg (Fig. 2) – and worsened the behavioral parameters (Table 2). In particular, the highest doses of i.p. L-NAME worsened intromission ability, whereas mounting frequency was greatly increased; this indicates that i.p. L-NAME neither impairs motor performance nor reduces sexual drive, but more specifically reduces intromission capacity. In contrast, the i.c.v. administration of L-NAME, up to the dose of 300 μg/rat, had no significant influence on the percentage of either mounting or ejaculating animals.

3.3. No synthase mRNA in defined brain nuclei

As shown in Figs. 3 and 4, NO synthase mRNA was expressed in all areas examined (bed nucleus of the stria terminalis, paraventricular nucleus of the hypothalamus, amygdala), the strongest labelling being observed in the medial amygdala. While the radioactivity in the bed nucleus of the stria terminalis and amygdala was practically the same in sexually potent and impotent rats, the expression of NO synthase mRNA in the magnocellular component of the paraventricular nucleus of sexually potent rats was approximately twice that observed in impotent ones.

4. Discussion

Our present data obtained following peripheral administration of either L-arginine or L-NAME are of rather straightforward interpretation. The injection of L-arginine increased the percentage of copulating and ejaculating subjects in the group of sexually inexperienced rats, whereas L-NAME had opposite effects. In the group of sexually experienced rats, where the percentage of copulating and ejaculating subjects was already 100%, the i.p. administration of L-arginine significantly shortened mount and intromission latencies and

Table 2
Influence of intraperitoneal administration of N^G-nitro-L-arginine (L-NAME) on male sexual behavior, in sexually experienced rats

Treatment (mg/kg)	ML (s)	IL (s)	EL (s)	MF	IF (No)	PEI (No)	ICI (s)	CE (s)
Saline	318.6 ± 132.4	363.2 ± 121.7	863.3 ± 187.4	3.8 ± 1.5	11.7 ± 2.3	503.0 ± 104.1	73.8	0.75
L-NAME, 10	524.3 ± 170.6	589.3 ± 167.0	1001.4 ± 142.3	5.2 ± 1.8	9.0 ± 2.1	629.2 ± 112.6	111.3	0.63
L-NAME, 25	614.5 ± 98.2 [a]	614.5 ± 98.2 [a]	1692.1 ± 208.7 [a]	6.4 ± 1.7	3.7 ± 1.2 [a]	895.2 ± 121.3 [a]	457.3	0.36 [a]
L-NAME, 50	477.1 ± 106.7	1353.9 ± 189.1 [a]	1650.3 ± 139.6 [a]	13.8 ± 6.7 [a]	4.6 ± 1.9 [a]	> 900 [a]	–	0.25 [a]

Groups of 10–13 animals. Treatment was given 60 min prior to the test. Each value is the mean ± S.E.M. [a] $P < 0.05$ compared with saline-treated controls (Mann-Whitney U-test). ML = mount latency; IL = intromission latency; EL = ejaculation latency; MF = mount frequency; IF = intromission frequency; PEI = post-ejaculatory interval; ICI = intercopulatory interval; CE = copulatory efficacy.

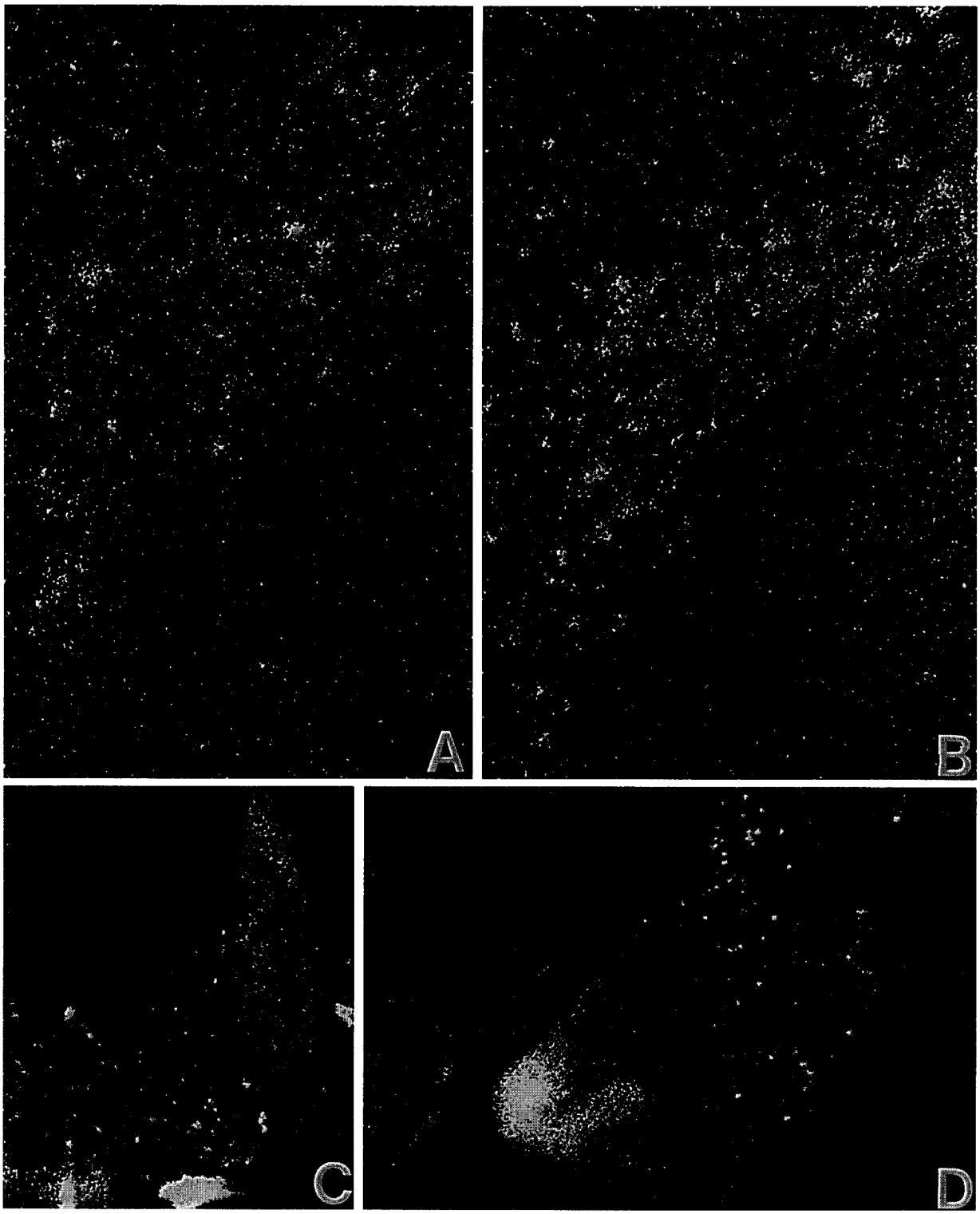

Fig. 4. NO synthase mRNA expression in the hypothalamic paraventricular nucleus of impotent (A) and potent (B) male rats. No differences are visible in the bed nucleus of stria terminalis (C) or in the amygdaloid complex (D).

post-ejaculatory interval, whereas L-NAME reduced the percentage of mounting and ejaculating subjects, significantly increased mount, intromission and ejaculation latencies, post-ejaculatory and intercopulatory intervals, and reduced intromission frequency and copulatory efficacy.

The behavioral data obtained following i.c.v. administration of either L-arginine or L-NAME are however

at variance. Indeed, in sexually experienced rats neither L-arginine nor L-NAME significantly modified any of the indexes of copulatory behavior, and in sexually inexperienced rats only L-NAME worsened – while L-arginine did not improve – sexual performance.

On the whole, our behavioral data indicate that NO plays an important role in the complex regulation of male sexual activity, and suggest that the main site(s) of action is/are at the peripheral level. This is consistent with the well-known, essential physiological role of NO as a peripheral mediator of a fundamental component of male copulatory activity – i.e. penile erection – by an action on the penile vasculature (Burnett et al., 1992; Anderson, 1993).

Nevertheless, a role for NO in male copulatory behavior, not only in the periphery level but also in the central nervous system (CNS), is suggested by our in situ hybridization data, showing that there is a striking difference between sexually potent and sexually impotent rats as regards NO synthase mRNA expression in a discrete neuronal population (magnocellular component of the paraventricular nucleus), its amount in sexually potent rats being twice that contained in the same nucleus of sexually impotent rats. The NO synthase mRNA expression in the parvocellular subdivision of the paraventricular nucleus was the same in the two groups of rats. This finding is of particular interest, since both magno- and parvocellular oxytocin-synthesizing neurons are highly concentrated in the paraventricular nucleus of the hypothalamus (Ivell, 1986), and an important physiological role for oxytocin in male sexual behavior has been demonstrated repeatedly (Arletti et al., 1985, 1990, 1992; Argiolas et al., 1988).

The inconsistency between behavioral data obtained following i.c.v. treatment with NO synthase substrate and inhibitor (which suggest that brain NO plays a minor role in male sexual activity) and hybridization data (which, in contrast, suggest an important role for NO in selected brain areas involved in male copulatory behavior) is not easy to interpret. It is of course possible that NO synthase in the paraventricular nucleus of the hypothalamus is of only minor importance in the complete expression of the whole male copulatory repertoire and in the complex CNS regulation of male sexual activity. It may also be that NO synthase activity in the paraventricular nucleus of the hypothalamus is more effectively modified following systemic administration of the natural substrate or of an inhibitor, rather than after their injection into the cerebrospinal fluid. In this connection, recent data have shown that the stimulatory effect of oxytocin, apomorphine and adrenocorticotrophic hormone on the occurrence of penile erection is indeed more effectively antagonized by the systemic than by the i.c.v. administration of NO synthase inhibitors (Melis and Argiolas, 1993; Poggioli et al., 1994).

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