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Role of Nitric Oxide Synthase Isoforms in Nitrous Oxide Antinociception in Mice

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ABBREVIATIONS: N_2O, nitrous oxide; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; s.c., subcutaneous; i.c.v., intracerebroventricular; i.t., intrathecal; SBP, systolic blood pressure; SMTC, S-methyl-L-thiocitrulline; L-NIO, L-N^5-(1-iminoethyl)ornithine; AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; EDTA, ethylenediazminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced; BH4, tetrahydrobiopterin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L-NAME, L-N^G-nitro arginine methyl ester; L-NOARG, L-N^G-nitro arginine; AD_{50}, analgesic dose, 50%; ANOVA, analysis of variance.

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ABSTRACT

Exposure of mice to the anesthetic gas N_2O evokes a prominent antinociceptive effect that is sensitive to antagonism by non-selective NOS inhibitors. The present study was conducted to identify whether a specific NOS isoform is implicated in N_2O antinociception in mice. In the abdominal constriction test, exposure of mice to 25%, 50% and 70% N_2O resulted in a concentration-dependent antinociceptive effect that persisted for up to six min following removal of the mice from the N_2O atmosphere into room air. This N_2O antinociceptive effect was antagonized by pretreatment with S-methyl-L-thiocitrulline (SMTC) and higher doses of L-N^5-(1-iminoethyl)ornithine (L-NIO), which reportedly inhibit the neuronal and endothelial isoforms of NOS, respectively. However, the N_2O-induced antinociception was unaffected by pretreatment with low doses of either SMTC or L-NIO or by pretreatment with 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), which selectively inhibits inducible NOS. S.c. pretreatment with SMTC and L-NIO reduced brain NOS activity in a dose-dependent manner, while AMT had no such effect. Moreover, in blood pressure experiments, SMTC increased SBP in dose-unrelated fashion, whereas L-NIO showed an appreciably weaker but dose-related increase in SBP. The i.c.v. pretreatment with SMTC also reduced N_2O antinociception and brain NOS activity without increasing of SBP. These results suggest that it is the neuronal isoform of NOS that is involved in mediation of the antinociceptive effect of N_2O in the mice.

N_2O is a widely used general anesthetic gas with many clinical applications, including enhancement of basal anesthesia by an inhalation anesthetic agent (*i.e*., the second gas effect) (Evers and Crowder, 2001), production of conscious sedation for dental surgery in anxious patients (Jackson and Johnson, 2002), and emergency relief of severe anxiety and pain (Kennedy and Luhmann, 2001). N_2O has also been used for patient-administered analgesia (Casatera et al., 2001), relief of labor pains (Rosen, 2002), pre-emptive analgesia (Katz, 1995) and reduction of pain and discomfort in various medical procedures, including intra-articular drug injection (Cleary et al., 2002), peripheral intravenous cannulation (Gerhardt et al., 2001), sigmoidoscopy (Harding and Gibson, 2000), colonoscopy (Forbes and Collins, 2000), ophthalmologic procedures (Cook et al., 2002) and biopsy procedures (Masood et al., 2002).

An involvement of endogenous opioid systems in N_2O-induced analgesia is evidenced by observations that N_2O antinociception in experimental animals was sensitive to antagonism by naloxone and other opioid receptor blockers (Berkowitz et al., 1976; Quock et al., 1990, 1993). There is also evidence that N_2O antinociception is secondary to stimulated neuronal release of endogenous opioid peptides (Quock et al., 1985; Zuniga et al., 1987).

Previous studies in our laboratory using the mouse abdominal constriction model have demonstrated that N_2O antinociception was antagonized in dose-related fashion by naloxone (Quock et al., 1993) and more specifically by selective κ-opioid receptor blockers (Quock et al., 1990). This was also verified by the failure of β-chlornaltrexamine to antagonize N_2O antinociception in mice, in which κ-opioid receptors were protected against alkylation by coadministration of a κ-opioid ligand

(Quock and Mueller, 1991). This implication of κ-opioid receptors is also consistent with recent reports that N_2O antinociception in mice is antagonized by i.c.v. and i.t. pretreatment with rabbit antisera against rat dynorphin (Branda et al., 2000; Cahill et al., 2000).

Previous findings from our laboratory also demonstrated that inhibition of NO synthesis antagonized N_2O antinociception in rats and mice (McDonald et al., 1994). NOS-inhibitors also attenuated the ability of i.c.v.-administered β-endorphin to stimulate the neuronal release of methionine-enkephalin in the rat spinal cord (Hara et al., 1995), suggesting that stimulated neuronal release of endogenous opioid peptides might be dependent on NO.

NO is synthesized as a by-product of conversion of its physiological precursor L-arginine to L-citrulline. This reaction is catalyzed by a family of enzymes known as NO synthase (NOS). There are two constitutive forms of the enzyme—neuronal NOS (nNOS) and endothelial NOS (eNOS)—and an inducible form, inducible NOS (iNOS). nNOS is classically found in central and peripheral neurons, where NO plays a role in neurotransmission and neuromodulation. eNOS is largely found in endothelial cells and has a substantial role in blood pressure regulation (Dominczak and Bohr, 1995). These two NOS isoforms are regulated by Ca^{2+} and calmodulin and constitutively expressed in tissues. In contrast, iNOS is widely distributed among immune cells, including macrophages and glial cells, is induced by various stimuli (*e.g.*, endotoxin) and is activated independent of Ca^{2+} (Jacobs et al., 1997). The recent development of compounds that possess relative selectivity for inhibiting different isoforms of NOS

allows identification of the specific NOS isoforms involved in specific physiological, pathological or pharmacological functions.

The aim of the present study was to utilize isoform-selective NOS inhibitors and determine whether their influences on N2O antinociception were consistent with an involvement of the neuronal form of NOS.

METHODS

Animals

Male NIH Swiss mice (20-33g) were purchased from Harlan Sprague Dawley Laboratories (Indianapolis, Indiana) and used in this research. Mice were housed five per cage with food and water available *ad libitum* in the AAALAC-accredited Wegner Hall Vivarium at Washington State University. The facility was maintained on a 12-h light/dark cycle (lights on 07:00-19:00) under standard conditions (22 ± 1°C room temperature, 33% humidity). Mice were kept in the holding room for at least four days following arrival in the facility. Animals were used only one time then discarded. This research was approved by the institutional animal care and use committee of Washington State University and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Antinociceptive testing in mice

Antinociception was assessed using the abdominal constriction test. The mice were treated i.p. with 0.1 ml per 10 g of body weight of 0.6 % glacial acetic acid in distilled water and immediately placed into the test chamber; exactly 5 min later, the number of abdominal constrictions—length-wise stretches of the torso with concave arching of the back—was counted for each mouse over a 6-min observation period. In prior training sessions, the numbers of abdominal constrictions recorded in test animals were very consistent between trained observers who were or were not aware of drug treatment. Consequently, the observer for the experiments in this study was not blind to the drug condition of the various groups of mice.

Groups of one to six mice each were exposed to N_2O inside an enclosed pre-filled Plexiglas box (35 cm length $\times$ 20 cm width $\times$ 15 cm height) with an airtight hinged lid. N_2O in O_2 was continuously delivered into the box using a standard dental sedation system (Porter, Hatfield, Pennsylvania). The amounts of N_2O and O_2 were varied within a total inflow rate of 10 liters/min to achieve the desired test concentration (25% N_2O: 2.5 liters/min N_2O and 7.5 liters/min O_2; 50% N_2O: 5.0 liters/min N_2O and 5.0 liters/min O_2; and 70% N_2O: 7.0 liters/min N_2O and 3.0 liters/min O_2). Gas entered the box through an inflow port at one end, circulated through the box and exited through an outflow port at the other end. Exhausted gases were vented from the box to a nearby fume hood. The concentrations of N_2O and O_2 in the box were continuously monitored using a POET II anesthetic monitoring system (Criticare, Milwaukee, Wisconsin). Control animals were exposed to room air in lieu of N_2O and O_2.

In most experiments of this study, the protocol consisted of an i.p. injection of acetic acid followed by a 5-min exposure to N_2O in the chamber then removal from the exposure chamber to a cage in room air, followed by a 6-min observation period, during which the number of abdominal constrictions was recorded. In room air-exposed mice, acetic acid-induced abdominal constrictions generally appear in 2-3 min, peaks during the 6-min observation period and slowly ebb with occasional constrictions occurring beyond 6 min.

To verify that the antinociceptive effect of N_2O was still in effect during the 6-min observation time, three additional groups of mice were tested under modified conditions. As represented in the left panel of Fig. 1, the atmosphere during the 6-min observation

period was varied from all-N_2O to all-room air with two intermediate states wherein mice were transferred from N_2O to room air 2 and 4 min into the 6-min observation period.

The antinociceptive effect of N_2O in different treatment groups of mice was quantified using the following formula:

$$\% \text{ antinociception} = 100 \times \frac{\#\text{ constrictions in control mice} - \#\text{ constrictions in exposed mice}}{\#\text{ constrictions in control mice}}$$

Separate vehicle-treated groups of mice were used as controls.

Assay of neuronal nitric oxide synthase activity

Mice were pretreated s.c. or i.c.v. with different doses of each NOS-inhibitor. After pretreatment times of 30 min for SMTC and L-NIO and 60 min for AMT, the mice were euthanized by decapitation for collection of cerebella following s.c. pretreatment or whole brains following i.c.v. pretreatment. NOS activity was determined in the cerebellar or brain homogenate by the conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline. The cerebellum or whole brain was homogenized in 2 volumes of Tris-HCl buffer (50 mM, pH 7.4) containing 2 mM EDTA and 2 mM EGTA, and centrifuged at 12,000 rpm at 4°C for 5 min. Twenty µl of supernatant were added to test tubes containing 50 mM Tris-HCl buffer, 10 mM NADPH, 6 mM $CaCl_2$, 6 mM BH4, 2 mM FAD, 2 mM FMN and 0.5 µCi [^{14}C]L-arginine monohydrochloride (Amersham Pharmacia Biotech, Piscataway, New Jersey) in a final volume of 40 µl at pH 7.4. Following incubation at 37°C for 30 min, the reaction was terminated by the addition of 50 mM HEPES buffer containing 5 mM EDTA and resin. Then the reaction mixture applied onto 1.5 ml columns of Dowex AG50WX-8. [^{14}C]L-citrulline was quantified by scintillation spectroscopy of 10-ml

aliquots of the flow-through. Protein concentration was determined using a standard protein assay kit (Pierce Chemical Company, Rockford, Illinois). NOS activity was expressed in terms of pmol citrulline formed/mg protein per min and then expressed as % of control.

Measurement of systolic blood pressures

Systolic blood pressure measurements were made non-invasively by the plethysmographic (tail-cuff) technique, using a Model 59 pulse amplifier and dual channel recorder (IITC Inc., Life Science, Woodland, California). Mice were anesthetized with an i.p. injection of ketamine (150 mg/kg) and xylazine (12.5 mg/kg). The mouse tail was inserted through the inflatable cuff of the sensor block, which also contained the photoelectric sensor and light source. The cuff was inflated to occlude the tail blood supply. As the pressure was slowly released, a sensitive pulse transducer detected the return of blood flow, and the “break-through“ SBP was determined from the strip chart. The tail-cuff measures were derived from the average of three measurements per animal. In NOS-inhibitor-treated mice, after a resting SBP was established (which generally required 15 min), mice received either s.c. or i.c.v. injections of SMTC or L-NIO. After 30 min pretreatment time, SBPs were determined and compared to the resting SBP.

Drugs

The following drugs were used in this research: N_2O, U.S.P. and O_2, U.S.P. (A & L Welding, Spokane, Washington); S-methyl-L-thiocitrulline (SMTC) (Sigma, St. Louis,

Missouri); 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT) (Sigma); and N(5)-(1-iminoethyl)-L-ornithine (L-NIO) (Alexis, San Diego, California).

For s.c. or i.p. pretreatments, drugs were administered in aqueous solution in injection volumes of 0.1 ml/10 g body weight. For i.c.v. injections, the mice were briefly anesthetized with halothane, U.S.P. (Halocarbon®, River Edge, New Jersey), and a volume of 5 µl of drug solution or vehicle was injected into the lateral cerebral ventricle using a hand-held 10-µl microsyringe (Hamilton, Reno, Nevada) at a point on the calvarium 1.0 mm lateral to and 2.0 mm caudal to bregma and to a depth of 2.5 mm from the skull surface.

Statistical analysis of data

In the abdominal constriction test, the AD_{50} values and 95% confidence intervals were determined and compared by the method of Litchfield and Wilcoxon (1949). In the chemical experiments, the significance of difference between treatment groups was determined by one-way analysis of variance (ANOVA) and a *post-hoc* Tukey test.

RESULTS

N_2O antinociception in mice

A 5-min exposure of mice to 70% N_2O caused a uniform reduction in the number of abdominal constrictions regardless of whether the N_2O exposure was terminated (*i.e.*, mice were removed from the N_2O chamber and transferred to a cage in room air) immediately after the 5-min exposure period, 2 min into the 6-min assessment period, 4 min into the 6-min assessment period, or throughout the 6 min assessment period. Fig. 1 shows that the level of antinociception is constant regardless of the duration of N_2O exposure.

Influence of isoform-selective NOS inhibitors on N_2O antinociception in mice

S.c. pretreatment with 10, 30 or 50 mg/kg of the nNOS-inhibitor SMTC antagonized N_2O antinociception in a dose-related manner, as indicated by a progressive rightward shift of the N_2O antinociception dose-response curve (Fig. 2). I.c.v. pretreatment with 1.0 μg SMTC per mouse also significantly attenuated N_2O antinociception (Fig. 3). S.c. pretreatment with 10 or 30 mg/kg of the eNOS-inhibitor L-NIO resulted in antagonism of N_2O antinociception only at the higher dose (Fig. 4). S.c. pretreatment with 1.0 mg/kg of the iNOS-inhibitor AMT failed to influence N_2O antinociception (Fig. 5). None of these NOS-inhibitors administered alone suppressed abdominal constrictions.

Table 1 compares the AD_{50} values for N_2O antinociception in the various treatment groups depicted in Figs. 2 through 5. There is a significant increase in the AD_{50} values for N_2O antinociception in treatment groups receiving any of two s.c. doses

of SMTC, the i.c.v. dose of SMTC or the higher dose of L-NIO. Conversely, there is no appreciable change in the AD_{50} values for N_2O antinociception in treatment groups receiving AMT or the lower dose of L-NIO.

Influence of NOS-inhibitors on cerebellar NOS activity in mice

S.c. pretreatment with increasing doses of SMTC and L-NIO resulted in significant and dose-dependent reduction of cerebellar NOS activity (Fig. 6). On the other hand, there was no significant effect of AMT on cerebellar NOS activity. I.c.v. pretreatment with 1.0 μg SMTC per mouse also significantly reduced whole brain NOS activity.

Influence of NOS-inhibitors on mean SBP

S.c. treatment with increasing doses of SMTC also significantly elevated the mean SBP, albeit not in dose-related fashion (Table 2). When SMTC was administered in an i.c.v. dose of 1.0 μg/mouse, there was no change in mean SBP when compared to the vehicle treatment group. On the other hand, s.c. treatment with L-NIO caused a dose-dependent increase in the mean SBP.

DISCUSSION

There is abundant evidence that NO participates in neurotransmission or a signal transduction role in the central nervous system. Previous studies have demonstrated that pharmacological manipulation of brain NO levels can have significant impact on a variety of neurologic and behavioral functions, including neuromodulation (Kiss, 2000), neuroendocrine regulation (Rivier, 2001), synaptic plasticity (Hölscher, 1997), behavior (McLeod et al., 2001), thermoregulation (Kamerman et al., 2002), food intake (Kamerman et al., 2002), pain (Luo and Cizkova, 2000) and neurotoxicity (Mohanakumar et al., 2002).

NOS catalyzes the five-electron oxidation of L-arginine to L-citrulline and the free radical NO. Three major isoforms of NOS have been described: neuronal NOS (nNOS), which is found predominantly in the brain; endothelial NOS (eNOS), which is found in vascular endothelium; and an inducible NOS (iNOS), which is present in activated macrophages. Inhibitors of NOS are invaluable tools in investigating physiological or pharmacological roles of NO, and extensive research has identified inhibitors with relative selectivity for each NOS isoform. SMTC is considered a potent nNOS inhibitor with a 10-fold selectivity for nNOS compared with eNOS and 28-fold for nNOS compared with iNOS (Furfine et al., 1994). L-NIO is approximately 8-fold more potent against eNOS than nNOS and 4-fold more potent against eNOS than iNOS (Rees et al., 1990; McCall et al., 1991). On the other hand, AMT is a selective inhibitor of iNOS, being 10-fold more potent against iNOS than nNOS and 42-fold more potent against iNOS than eNOS (Tracey et al., 1995; Nakane et al., 1995).

Consistent with many earlier reports from our laboratory (Quock et al., 1990, 1993; Quock and Mueller, 1991; McDonald et al., 1994), exposure to N_2O evoked a concentration-related antinociception in mice. The current findings also demonstrate that a 5-min exposure to N_2O suppresses abdominal constrictions for at least 6 min following termination of N_2O exposure and removal to room air. Hence, it would appear that continuous inhalation of N_2O is not necessary for continued antinociception. N_2O must, therefore, activate a mechanism or cascade that continues to its ultimate conclusion (antinociception) despite termination of exposure and the rapid elimination of N_2O from the body in the expired air. That central mechanism is hypothesized to be the stimulated neuronal release of dynorphin with subsequent activation of central κ-opioid receptors (Branda et al., 2000; Cahill et al., 2000).

The N_2O-induced antinociceptive effect was dose-dependently antagonized by SMTC and L-NIO but not AMT. The antagonism was in agreement with earlier findings of an inhibitory effect of the non-selective NOS inhibitors L-NAME and L-NOARG on N_2O antinociception (McDonald et al., 1994). Superficially, these findings implicate the neuronal and endothelial isoforms of NOS in N_2O antinociception. Since most NO in the brain likely results from an action of nNOS, the question is whether the eNOS of the cerebral vasculature might be involved in N_2O antinociception.

First, we measured NOS activity in the cerebellum as a general index of nNOS activity after treatment of each NOS inhibitor. As expected, SMTC, a selective nNOS-inhibitor, reduced the cerebellar NOS activity in a dose-dependent manner. AMT is reportedly a selective inhibitor of iNOS (Nakane et al., 1995). Despite a recent study questioning its selectivity (Boer et al., 2000), it also had no effect on cerebellar NOS

activity at a dose that significantly reduced the lipopolysaccharide-induced increase in plasma nitrites and nitrates (Tracey et al., 1995). Ostensibly selective for eNOS, L-NIO also reduced cerebellar NOS activity in a dose-related manner. Low dose L-NIO (10 mg/kg) had no effect on N_2O antinociception and no effect on cerebellar NOS activity. However, high dose L-NIO (30 mg/kg) antagonized N_2O antinociception and inhibited cerebellar NOS activity. This is likely the result of loss of selectivity for eNOS and emerging inhibition of nNOS at higher doses (30 mg/kg).

Secondly, we attempted to identify an appropriate index of eNOS activity. Attempts to measure NOS activity in descending aorta were fraught with difficulty because of limited tissue mass and low levels of NOS activity. Since eNOS is largely found in endothelial cells and is thought to have a substantial role in blood pressure regulation (Dominiczak and Bohr, 1995), it was thought that SBP might be sensitive to changes in eNOS activity. It is known that inhibition of eNOS can induce an increase in SBP (Rees et al., 1989). In the present study, both SMTC and L-NIO elevated mean SBP. Although SMTC was previously characterized as a selective inhibitor of the nNOS isoform (Furfine et al., 1994), all three doses of SMTC significantly increased the mean SBP, which was consistent with earlier reports that SMTC elevates SBP (Narayanan et al., 1995). This effect has been explained on the basis of possible inhibition of nNOS in cardiovascular regulating regions of the brain (Ollerstam et al., 1997), but detailed mechanisms remain unclear. These SBP-increasing effects of SMTC were not dose-related and were not correlated to antagonism of N_2O antinociception in a dose-dependent manner as did inhibition of cerebellar NOS activity.

In the present study, L-NIO caused significant dose-related increases in the mean SBP. However, this SBP-elevating effect of L-NIO was weaker than that of SMTC. Although previous reports showed that L-NIO increased SBP significantly, that effect was ten times weaker than the other arginine analogue inhibitors (L-NAME or L-NOARG) (Rees et al., 1990). In our preliminary experiments, the SMTC effect was comparable to that of L-NOARG. Therefore, the present results are consistent with the results of earlier studies.

In addition to experiments employing systemic pretreatment with NOS-inhibitors, SMTC was also introduced directly into the brain. SMTC, administered via the i.c.v. route, produced a significant antagonism of N_2O antinociception. This pretreatment also significantly cerebellar NOS activity without causing an elevation in mean SBP. In earlier research, L-NAME, a non-selective NOS-inhibitor, was also found to be an effective antagonist of N_2O antinociception following i.c.v. pretreatment (McDonald et al., 1994).

In conclusion, N_2O antinociception in the mouse abdominal constriction test was most effectively antagonized by the selective nNOS-inhibitor SMTC in a dose-dependent manner. This antagonism of antinociception was dose-dependently correlated with inhibition of cerebellar NOS activity but not with increasing SBP. At low doses, the selective eNOS-inhibitor L-NIO was ineffective in antagonizing N_2O antinociception or inhibiting cerebellar NOS activity. A higher dose of L-NIO not only reduced N_2O antinociception but also inhibited cerebellar NOS activity. L-NIO also caused a dose-related increase in SBP. It is presumed that, at high doses, L-NIO loses its selectivity for eNOS and also affects nNOS. The selective iNOS-inhibitor AMT was

ineffectual in antagonizing N_2O antinociception and also failed to reduce cerebellar NOS activity. These results suggest that the neuronal isoform of NOS is involved in mediation of the antinociceptive effect of N_2O in the mouse.

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FOOTNOTES

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TABLE 1. The influence of pretreatment with NOS-inhibitors on AD_{50} values for N_2O antinociception in the mouse abdominal constriction test

Treatment Group	AD_{50} Value for N_2O Antinociception (95% Confidence Intervals)
<u>Systemic pretreatment</u>	
Vehicle (s.c.) + N_2O	68.1% (32.8-141.5%)
SMTC (10 mg/kg, s.c.) + N_2O	51.2% (27.5-95.3%)
SMTC (30 mg/kg, s.c.) + N_2O	129.8% (86.0-195.9%) ✳
SMTC (50 mg/kg, s.c.) + N_2O	202.5% (133.2-307.8%) ✳
L-NIO (10 mg/kg, s.c.) + N_2O	92.0% (51.6-164.0%)
L-NIO (30 mg/kg, s.c.) + N_2O	112.2% (83.2-151.2%) ✳
AMT (1.0 mg/kg, s.c.) + N_2O	48.9% (34.6-69.2%)
<u>Central pretreatment</u>	
Vehicle (i.c.v.) + N_2O	51.0% (33.0-78.8%)
SMTC (1.0 μg/mouse, i.c.v.) + N_2O	105.4% (72.1-153.9%) ✳, #

Significance of difference: ✳, $P < 0.05$ vs. vehicle (s.c.) treatment; #, $P < 0.05$ vs. vehicle (i.c.v.) treatment.

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TABLE 2. The influence of pretreatment with NOS-inhibitors on SBP in mice

Dose	Mean Systolic Blood Pressure (mm Hg)	
	0 min	30 min
Vehicle	66.5 ± 9.7	77.1 ± 13.6
SMTC (10 mg/kg, s.c.)	63.5 ± 10.7	134.9 ± 9.8 ✳✳✳
SMTC (30 mg/kg, s.c.)	67.5 ± 6.7	138.4 ± 11.5 ✳✳✳
SMTC (50 mg/kg, s.c.)	67.6 ± 2.3	145.9 ± 11.0 ✳✳✳
SMTC (1.0 μg/mouse, i.c.v.)	66.7 ± 5.1	70.9 ± 4.6
L-NIO (10 mg/kg, s.c.)	65.1 ± 7.3	103.2 ± 16.1 ✳✳✳
L-NIO (30 mg/kg, s.c.)	68.6 ± 9.5	128.4 ± 26.2 ✳✳✳, ###

Values represent the mean SBP of 12-24 mice per group. Significance of difference: ✳✳✳, $P < 0.001$ vs. vehicle control; ###, $P < 0.001$ vs. L-NIO (10 mg/kg).

LEGENDS TO FIGURES

Fig. 1. Effect of various durations of N_2O exposure in the mouse abdominal constriction test. Data represent the mean % antinociceptive response of 12-18 mice per group.

Fig. 2. Dose-response curves for N_2O antinociception in the mouse abdominal constriction test following s.c. pretreatment with SMTC: ○, vehicle (control); ◆, 10 mg/kg; ●, 30 mg/kg; and ■, 50 mg/kg. Symbols represent the mean % antinociceptive response in 9-22 mice per treatment group.

Fig. 3. Dose-response curves for N_2O antinociception in mice test following i.c.v. pretreatment with SMTC: ○, vehicle (control); and ●, 1.0 μg/mouse. Symbols represent the mean % antinociceptive response in 10-12 mice per treatment group.

Fig. 4. Dose-response curves for N_2O antinociception in mice following s.c. pretreatment with L-NIO: ○, vehicle (control); ◆, 10 mg/kg; and ●, 30 mg/kg. Symbols represent the mean % antinociceptive response in 12-24 mice per treatment group.

Fig. 5. Dose-response curves for N_2O antinociception in mice following s.c. pretreatment with AMT: ○, vehicle (control); and ▲, 1.0 mg/kg. Symbols represent the mean % antinociceptive response in 10-18 mice per treatment group.

Fig. 6. Influence of pretreatment with NOS-inhibitors on cerebellar and whole brain NOS activity in mice. Bars and vertical lines represent the mean and standard errors of the mean (s.e.m.) of 5-33 mice per treatment group. NOS activity for each treatment group is expressed as % of the control group, where the s.c. control was 29.3 ± 1.6 and the i.c.v. control was 20.4 ±0.6 pmol citrulline formed/mg protein per min. Significance of difference: ***, P < 0.001 vs. control; ##, ###, P < 0.01, 0.001 vs. SMTC (10 mg/kg).

Fig. 1

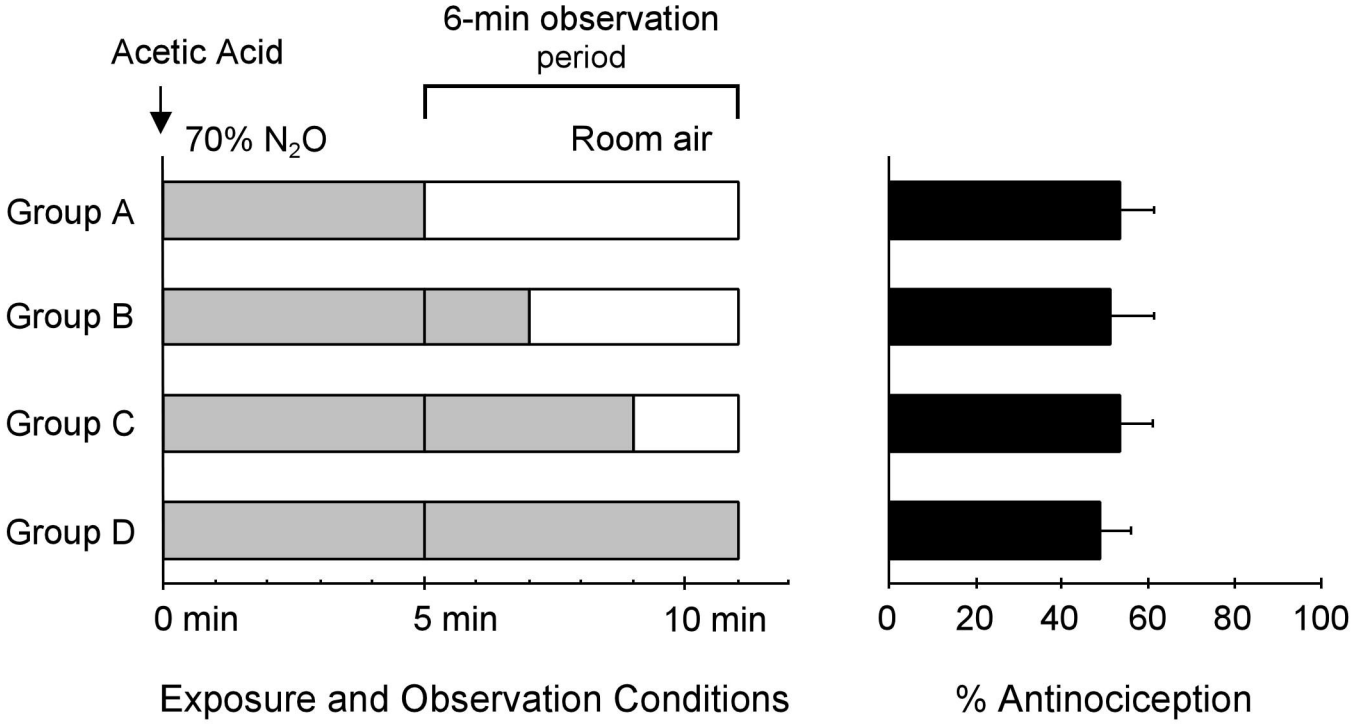

6-min observation
period
Acetic Acid
70% N_2O
Room air
Group A
Group B
Group C
Group D
0 min
5 min
10 min
Exposure and Observation Conditions
0
20
40
60
80
100
% Antinociception

Fig. 2

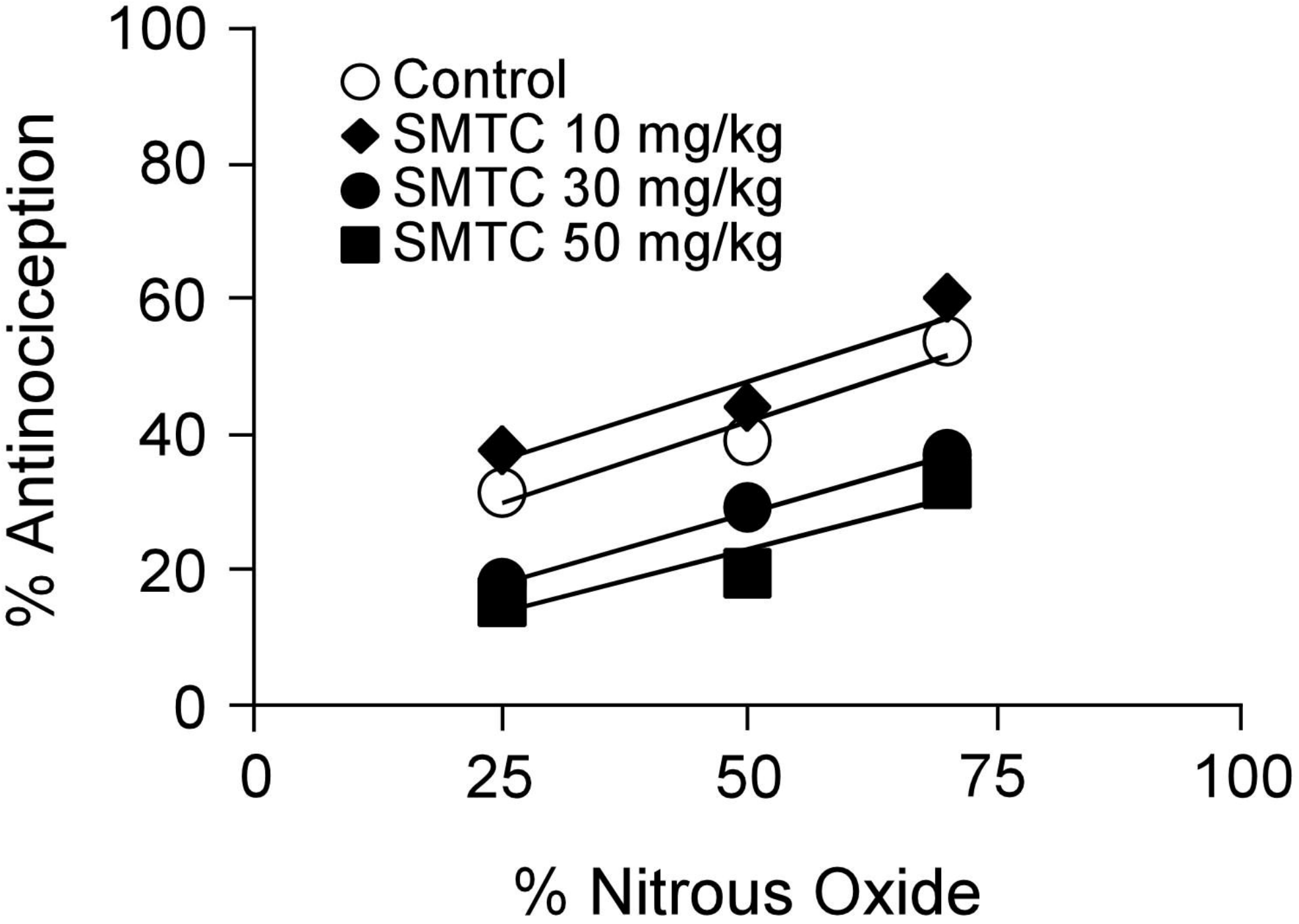

100
80
60
40
20
0
% Antinociception
Control
SMTC 10 mg/kg
SMTC 30 mg/kg
SMTC 50 mg/kg
0
25
50
75
100
% Nitrous Oxide

Fig. 3

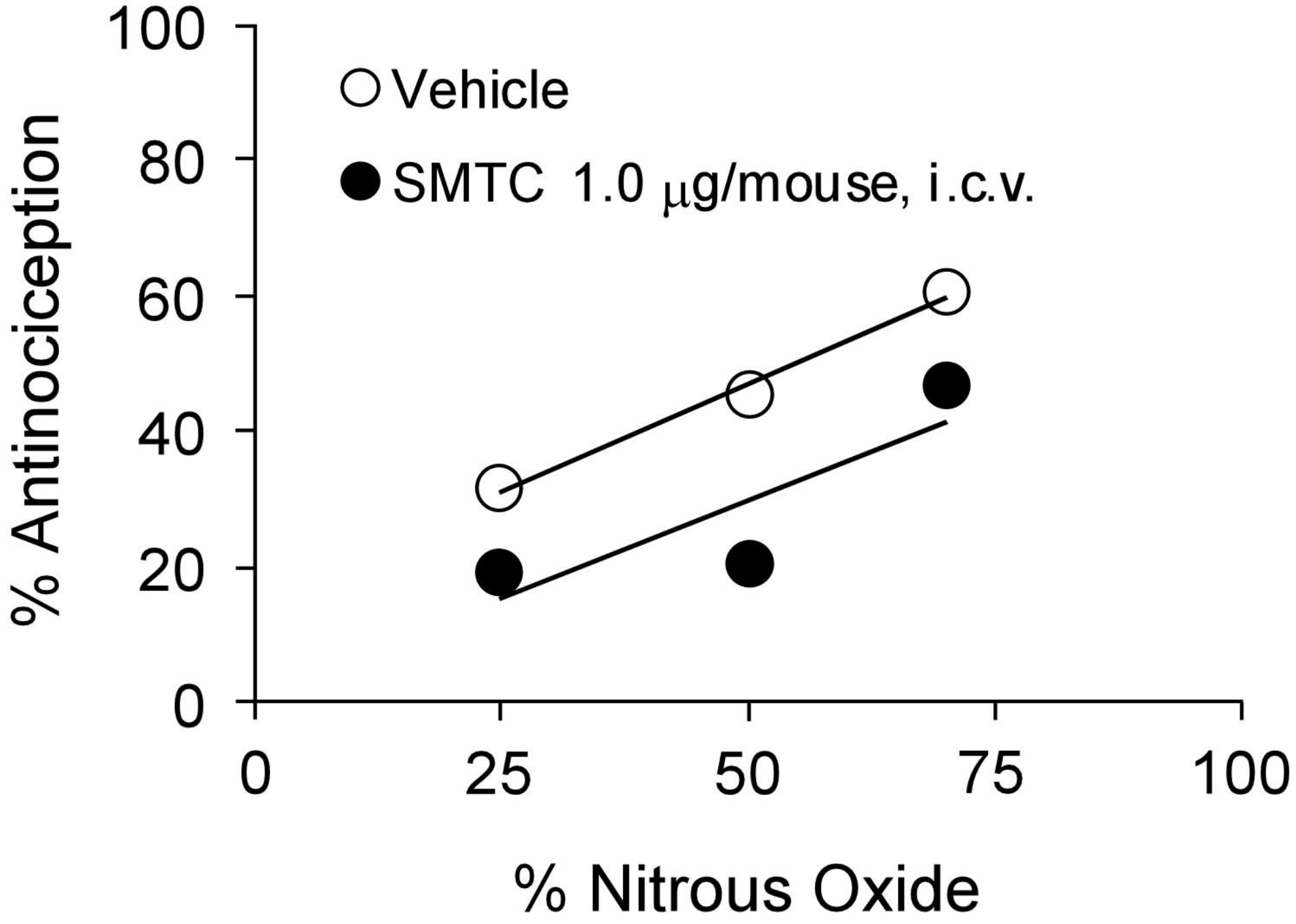

100
80
60
40
20
0
% Antinociception
Vehicle
SMTC 1.0 μg/mouse, i.c.v.
0
25
50
75
100
% Nitrous Oxide

Fig. 4

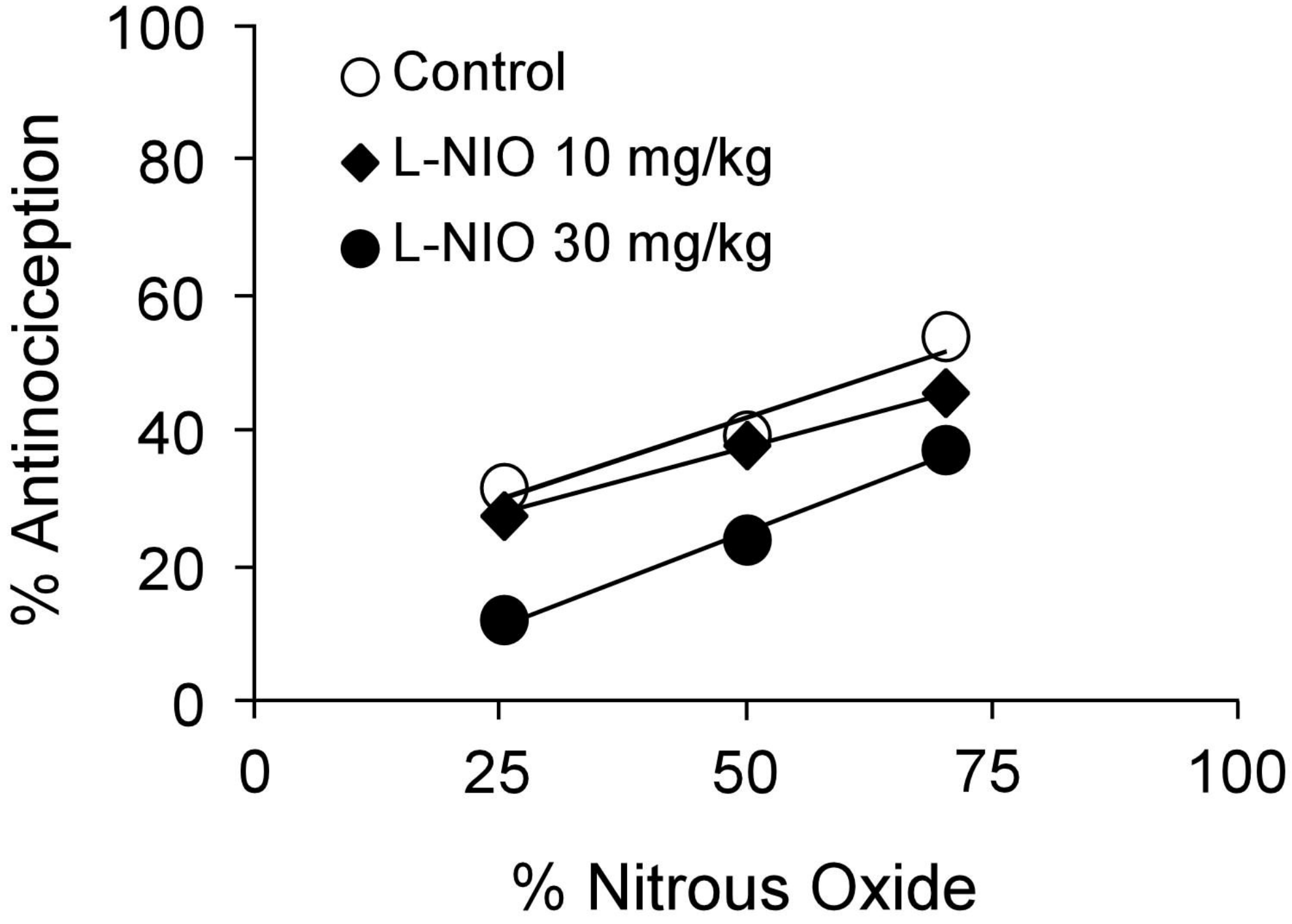

100
80
60
40
20
0
% Antinociception
Control
L-NIO 10 mg/kg
L-NIO 30 mg/kg
0
25
50
75
100
% Nitrous Oxide

Fig. 5

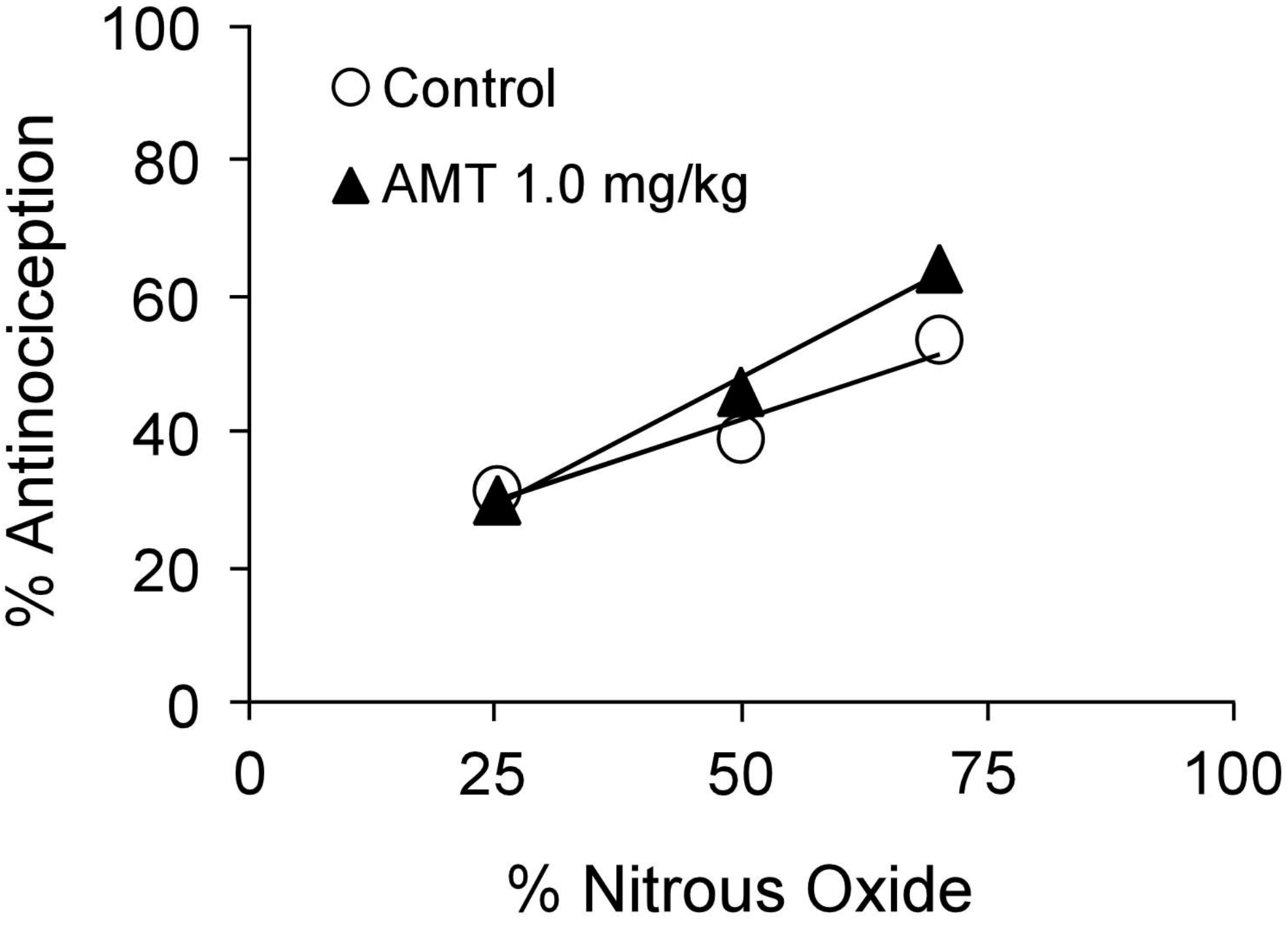
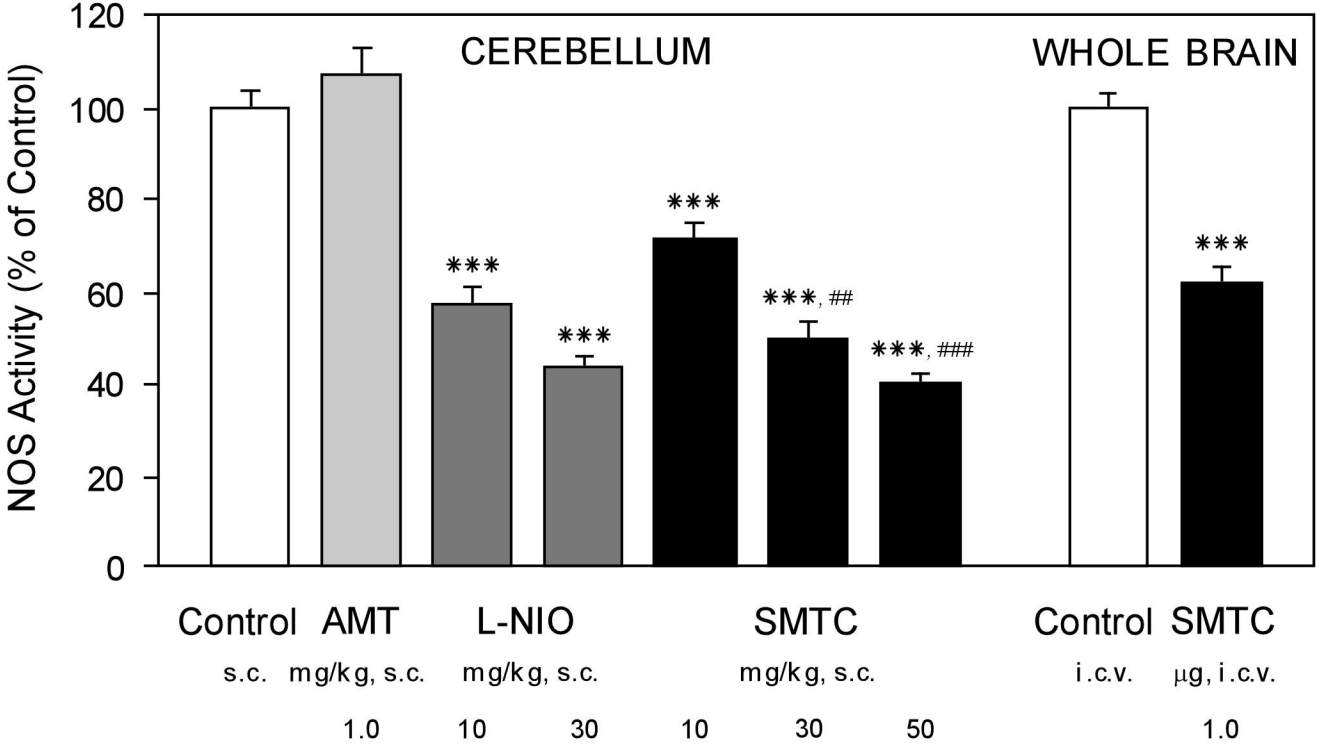

100
80
60
40
20
0
% Antinociception
Control
AMT 1.0 mg/kg
0
25
50
75
100
% Nitrous Oxide

Fig. 6


NOS Activity (% of Control)
120
100
80
60
40
20
0
CEREBELLUM
WHOLE BRAIN
***
***
***
***, ##
***, ###
***
Control
AMT
L-NIO
SMTC
Control
SMTC
s.c.
mg/kg, s.c.
mg/kg, s.c.
mg/kg, s.c.
i.c.v.
μg, i.c.v.
1.0
10
30
10
30
50
1.0