Role of Nitric Oxide Synthase Isoforms in Nitrous Oxide

Antinociception in Mice

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ABBREVIATIONS: N₂O, 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-Ν⁵-(1-iminoethyl)ornithine; AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic 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-Ν⁶-nitro arginine methyl ester; L-NOARG, L-Ν⁶-nitro arginine; AD₅₀, analgesic dose, 50%; ANOVA, analysis of variance.

Recommended section assignment: NEUROPHARMACOLOGY
ABSTRACT

Exposure of mice to the anesthetic gas N₂O 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 N₂O antinociception in mice. In the abdominal constriction test, exposure of mice to 25%, 50% and 70% N₂O resulted in a concentration-dependent antinociceptive effect that persisted for up to six min following removal of the mice from the N₂O atmosphere into room air. This N₂O antinociceptive effect was antagonized by pretreatment with S-methyl-L-thiocitrulline (SMTC) and higher doses of L-N⁵-(1-iminoethyl)ornithine (L-NIO), which reportedly inhibit the neuronal and endothelial isoforms of NOS, respectively. However, the N₂O-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 N₂O 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 N₂O in the mice.
N₂O 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). N₂O 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 N₂O-induced analgesia is evidenced by observations that N₂O 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 N₂O 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 N₂O 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 N₂O 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 N₂O 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 N₂O 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 Ca²⁺ 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 Ca²⁺ (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\textsubscript{2}O inside an enclosed pre-filled Plexiglas box (35 cm length \times 20 cm width \times 15 cm height) with an airtight hinged lid. N\textsubscript{2}O in O\textsubscript{2} was continuously delivered into the box using a standard dental sedation system (Porter, Hatfield, Pennsylvania). The amounts of N\textsubscript{2}O and O\textsubscript{2} were varied within a total inflow rate of 10 liters/min to achieve the desired test concentration (25\% N\textsubscript{2}O: 2.5 liters/min N\textsubscript{2}O and 7.5 liters/min O\textsubscript{2}; 50\% N\textsubscript{2}O: 5.0 liters/min N\textsubscript{2}O and 5.0 liters/min O\textsubscript{2}; and 70\% N\textsubscript{2}O: 7.0 liters/min N\textsubscript{2}O and 3.0 liters/min O\textsubscript{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\textsubscript{2}O and O\textsubscript{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\textsubscript{2}O and O\textsubscript{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\textsubscript{2}O 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\textsubscript{2}O 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-N₂O to all-room air with two intermediate states wherein mice were transferred from N₂O to room air 2 and 4 min into the 6-min observation period.

The antinociceptive effect of N₂O 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 [¹⁴C]L-arginine to [¹⁴C]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 CaCl₂, 6 mM BH₄, 2 mM FAD, 2 mM FMN and 0.5 µCi [¹⁴C]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. [¹⁴C]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: N2O, U.S.P. and O2, 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\textsubscript{2}O antinociception in mice

A 5-min exposure of mice to 70% N\textsubscript{2}O caused a uniform reduction in the number of abdominal constrictions regardless of whether the N\textsubscript{2}O exposure was terminated (i.e., mice were removed from the N\textsubscript{2}O 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\textsubscript{2}O exposure.

Influence of isoform-selective NOS inhibitors on N\textsubscript{2}O antinociception in mice

S.c. pretreatment with 10, 30 or 50 mg/kg of the nNOS-inhibitor SMTC antagonized N\textsubscript{2}O antinociception in a dose-related manner, as indicated by a progressive rightward shift of the N\textsubscript{2}O antinociception dose-response curve (Fig. 2). I.c.v. pretreatment with 1.0 µg SMTC per mouse also significantly attenuated N\textsubscript{2}O antinociception (Fig. 3). S.c. pretreatment with 10 or 30 mg/kg of the eNOS-inhibitor L-NIO resulted in antagonism of N\textsubscript{2}O 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\textsubscript{2}O antinociception (Fig. 5). None of these NOS-inhibitors administered alone suppressed abdominal constrictions.

Table 1 compares the AD\textsubscript{50} values for N\textsubscript{2}O antinociception in the various treatment groups depicted in Figs. 2 through 5. There is a significant increase in the AD\textsubscript{50} values for N\textsubscript{2}O 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$_2$O 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 N₂O evoked a concentration-related antinociception in mice. The current findings also demonstrate that a 5-min exposure to N₂O suppresses abdominal constrictions for at least 6 min following termination of N₂O exposure and removal to room air. Hence, it would appear that continuous inhalation of N₂O is not necessary for continued antinociception. N₂O must, therefore, activate a mechanism or cascade that continues to its ultimate conclusion (antinociception) despite termination of exposure and the rapid elimination of N₂O 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 N₂O-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 N₂O antinociception (McDonald et al., 1994). Superficially, these findings implicate the neuronal and endothelial isoforms of NOS in N₂O 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 N₂O 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 N₂O antinociception and no effect on cerebellar NOS activity. However, high dose L-NIO (30 mg/kg) antagonized N₂O 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 N₂O 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 N₂O 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 N₂O antinociception following i.c.v. pretreatment (McDonald et al., 1994).

In conclusion, N₂O 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 N₂O antinociception or inhibiting cerebellar NOS activity. A higher dose of L-NIO not only reduced N₂O 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$_2$O 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$_2$O in the mouse.
REFERENCES


FOOTNOTES

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TABLE 1. The influence of pretreatment with NOS-inhibitors on AD$_{50}$ values for N$_2$O antinociception in the mouse abdominal constriction test

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>AD$_{50}$ Value for N$_2$O Antinociception (95% Confidence Intervals)</th>
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<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Systemic pretreatment</strong></td>
<td></td>
</tr>
<tr>
<td>Vehicle (s.c.) + N$_2$O</td>
<td>68.1% (32.8-141.5%)</td>
</tr>
<tr>
<td>SMTC (10 mg/kg, s.c.) + N$_2$O</td>
<td>51.2% (27.5-95.3%)</td>
</tr>
<tr>
<td>SMTC (30 mg/kg, s.c.) + N$_2$O</td>
<td>129.8% (86.0-195.9%) *</td>
</tr>
<tr>
<td>SMTC (50 mg/kg, s.c.) + N$_2$O</td>
<td>202.5% (133.2-307.8%) *</td>
</tr>
<tr>
<td>L-NIO (10 mg/kg, s.c.) + N$_2$O</td>
<td>92.0% (51.6-164.0%)</td>
</tr>
<tr>
<td>L-NIO (30 mg/kg, s.c.) + N$_2$O</td>
<td>112.2% (83.2-151.2%) *</td>
</tr>
<tr>
<td>AMT (1.0 mg/kg, s.c.) + N$_2$O</td>
<td>48.9% (34.6-69.2%)</td>
</tr>
<tr>
<td><strong>Central pretreatment</strong></td>
<td></td>
</tr>
<tr>
<td>Vehicle (i.c.v.) + N$_2$O</td>
<td>51.0% (33.0-78.8%)</td>
</tr>
<tr>
<td>SMTC (1.0 µg/mouse, i.c.v.) + N$_2$O</td>
<td>105.4% (72.1-153.9%) * #</td>
</tr>
</tbody>
</table>

Significance of difference: *, P < 0.05 vs. vehicle (s.c.) treatment; #, P < 0.05 vs. vehicle (i.c.v.) treatment.
TABLE 2. The influence of pretreatment with NOS-inhibitors on SBP in mice

<table>
<thead>
<tr>
<th>Dose</th>
<th>Mean Systolic Blood Pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Vehicle</td>
<td>66.5 ± 9.7</td>
</tr>
<tr>
<td>SMTC (10 mg/kg, s.c.)</td>
<td>63.5 ± 10.7</td>
</tr>
<tr>
<td>SMTC (30 mg/kg, s.c.)</td>
<td>67.5 ± 6.7</td>
</tr>
<tr>
<td>SMTC (50 mg/kg, s.c.)</td>
<td>67.6 ± 2.3</td>
</tr>
<tr>
<td>SMTC (1.0 µg/mouse, i.c.v.)</td>
<td>66.7 ± 5.1</td>
</tr>
<tr>
<td>L-NIO (10 mg/kg, s.c.)</td>
<td>65.1 ± 7.3</td>
</tr>
<tr>
<td>L-NIO (30 mg/kg, s.c.)</td>
<td>68.6 ± 9.5</td>
</tr>
</tbody>
</table>

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 N₂O 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 N₂O antinociception in the mouse abdominal constriction test following s.c. pretreatment with SMTC: O, 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 N₂O antinociception in mice test following i.c.v. pretreatment with SMTC: O, 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 N₂O antinociception in mice following s.c. pretreatment with L-NIO: O, 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 N₂O antinociception in mice following s.c. pretreatment with AMT: O, 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. 2

The graph shows the relationship between the percentage of nitrous oxide and the percentage of antinociception for different SMTC doses. The control group is represented by open circles, SMTC 10 mg/kg by filled diamonds, SMTC 30 mg/kg by filled circles, and SMTC 50 mg/kg by filled squares. As the percentage of nitrous oxide increases, the percentage of antinociception also increases for all groups, but the effect is more pronounced at higher SMTC doses.
Fig. 6

![Bar chart showing NOS activity (% of control) in different parts of the brain.](chart.png)