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Nitrous Oxide Activates GABAergic Neurons in the Spinal Cord in Fischer Rats

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Background: **Findings to date indicate that nitrous oxide exerts its antinociceptive effect by activating descending noradrenergic neurons. The mechanism whereby descending inhibitory neurons, including noradrenergic neurons, produce antinociceptive effect remains unclear. Using c-Fos protein as a marker for neuronal activation, we examined whether spinal cord neurons activated by nitrous oxide are γ-aminobutyric acid–mediated (GABAergic) neurons.**

Methods: **Adult male Fischer (a strain in which nitrous oxide shows strong antinociceptive properties) and Lewis (a strain in which nitrous oxide lacks antinociceptive properties) rats were exposed to either air (control) or nitrous oxide. Frozen sections of the spinal cord were either stained for c-Fos or double-stained for c-Fos and glutamic acid decarboxylase (a rate-limiting enzyme for GABA synthesis) and analyzed by standard or confocal microscopy.**

Results: **In Fischer rats, 90 min of 75% N_2O administration increased the number of c-Fos–positive cells in the spinal cord approximately threefold as compared with the control group. The c-Fos–positive cells induced by nitrous oxide were almost entirely colocalized with glutamic acid decarboxylase–positive cells. In contrast, exposure did not change the number of c-Fos–positive cells in the spinal cord in Lewis rats.**

Conclusions: **Exposure to nitrous oxide activates GABAergic neurons in the spinal cord. The dose-dependence of GABAergic neuronal activation in the Fischer rats and its absence in the Lewis rat correlate with antinociceptive responses previously reported in these same circumstances. Together, we interpret these data to indicate that activation of GABAergic neurons in the spinal cord are involved in the antinociceptive action of nitrous oxide.**

NITROUS oxide has been used in clinical anesthesia for more than 150 years and remains one of the most commonly used drugs. Although the anesthetic mechanisms of nitrous oxide remain largely unclear, its analgesic and antinociceptive mechanisms have been partly revealed by recent studies.[1] Namely, nitrous oxide induces opioid peptide release in the periaqueductal gray area (PAG) of the midbrain leading to activation of descending noradrenergic neurons, which results in modulation of nociceptive processing in the spinal cord. Support for this hypothesis mainly stems from the following findings: (1) Systemic administration of opiate receptor antagonist blocks the antinociceptive effect of nitrous oxide in humans,[2,3] rats,[4–6] and mice[7,8]; (2) bilateral microinjection of opiate receptor antagonists into the ventrolateral PAG blocks the antinociceptive effect of nitrous oxide in rats,[5,9] whereas intrathecal injection of opiate receptor antagonist is without effect in rats[10] and mice[8]; (3) ablation of the PAG attenuates the antinociceptive effect of nitrous oxide in rats[11]; (4) intrathecally but not supraspinally administered α_2-adrenoceptor antagonist blocks the antinociceptive effect of nitrous oxide in rats[10]; (5) transection of the spinal cord eliminates the antinociceptive effect of nitrous oxide in rats[12]; (6) nitrous oxide provokes release of norepinephrine in the spinal cord in rats, and when norepinephrine is depleted, nitrous oxide is no longer able to produce its antinociceptive effect[12]; (7) when noradrenergic neurons of the brainstem (*i.e.,* A5, A6 [locus ceruleus], and A7) are chemically destroyed in rats, the animal no longer shows the antinociceptive effect of nitrous oxide[13]; and (8) α_{2B}-adrenoceptor subtype knockout mice do not exhibit antinociceptive properties of nitrous oxide.[13]

Involvement of descending inhibitory neurons in the antinociceptive effect of nitrous oxide was first suggested by an electrophysiologic study using decerebrate nonanesthetized cats in 1981.[14] Assuming that α_2 and α_1 adrenoceptors mediate inhibitory and excitatory neuronal activities, respectively, there are at least two neuronal systems that may be involved in the antinociceptive effect of nitrous oxide at the spinal cord level (fig. 1). One is the direct presynaptic inhibition of the nociceptive primary afferent neurons or postsynaptic inhibition of the second-order neurons through activation of the α_2 adrenoceptors; the other is the indirect activation of inhibitory interneurons through α_1 adrenoceptors. Involvement of α_2 adrenoceptors in the former is supported by several lines of experiments, including pharmacologic study, as previously mentioned,[10] and those using knockout mice.[13] In the spinal cord, γ-aminobutyric acid (GABA) and glycine are the two major inhibitory neurotransmitters that mediate fast synaptic inhibition. In the current study, we investigated the possible involvement of GABAergic neurons using c-Fos as a marker for neuronal pathways activated by nitrous oxide. First, we sought to characterize the expression pat-

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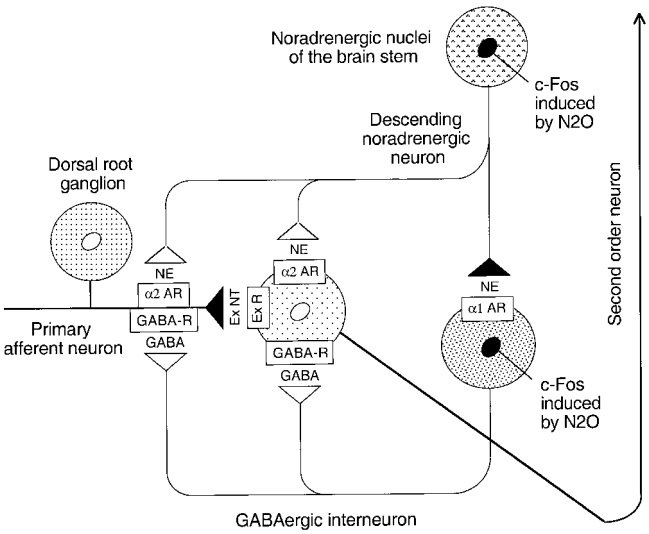

Fig. 1. Hypothetical neuronal pathways in the spinal cord involved in the antinociceptive effect of nitrous oxide. A closed triangle indicates an excitatory synapse, and an open triangle indicates an inhibitory synapse. AR = adrenoceptor; ExNT = excitatory neurotransmitters; ExR = receptors for excitatory neurotransmitters; GABA-R = GABA receptor; NE = norepinephrine.

tern of c-Fos in the spinal cord after nitrous oxide administration in two different strains of rats (*i.e.*, Fischer and Lewis strains); previously, we have demonstrated that nitrous oxide shows strong antinociceptive effect in the former strain and almost no effect in the latter.[15] Second, we sought to identify whether the type of neuron that was activated by nitrous oxide was GABAergic using double-staining analysis.

Materials and Methods

Animals

Adult male Fischer and Lewis rats were used throughout this study (B&K Universal, Grimston Aldbrough Hull, UK). The study protocol of the animal experiments was approved by the Home Office of the United Kingdom (London, UK), and all efforts were made to minimize animal suffering and reduce the number of animals used.

Nitrous Oxide Exposure

Gas exposure was performed in a Plexiglas chamber (45 cm long, 22.5 cm wide, and 20 cm high). A mixture of nitrous oxide and oxygen gas and air was continuously delivered from an anesthetic machine into the chamber through an inflow port, which was exhausted through an outflow port following through the Anesthetic Gas Scavenging System (Ohmeda, Louisville, CO). Gas flow rate was 10 l/min. Gas concentrations, including those for nitrous oxide, oxygen, and carbon dioxide, in the chamber were measured continuously by infrared gas spectrometry (Ohmeda 5250 RGM).

Spinal Cord Preparation and Cryosection

After exposure either to nitrous oxide or to air (control), animals were injected intraperitoneally with 100 mg/kg sodium pentobarbital. After being deeply anesthetized, the animals had thoracotomy by transverse incision at the level of diaphragm and midline sternotomy and were perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer through a 16-gauge cannula inserted through the left ventricle into the ascending aorta. (An incision was made in the right ventricle for drainage.) Animals were decapitated, and the entire spinal cord was expelled from the spinal canal by rapid injection of PBS at the sacral vertebral level. The spinal cord was stored in 30% sucrose in 0.1 M phosphate buffer for at least 24 h at 4°C. A portion of the spinal cord at the lumbar level (*i.e.,* approximately 5-mm length) was cut by a razor blade and freeze-mounted in embedding matrix, and 30-μm transverse sections at approximately L5 level were cut under −15°C, which were collected in PBS as free-floating sections.

Immunohistochemistry

Diaminobenzidine Staining of c-Fos. Free-floating spinal cord sections were first incubated for 1 h in blocking solution consisted of 3% rabbit serum and 0.3% Triton X in PBS (PBT). They were incubated overnight with goat anti–c-Fos antibody (1:10,000, catalog No. sc-52-G, Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution consisting 1% normal rabbit serum in PBS on a shaker at room temperature. The sections were rinsed with 1% rabbit serum in PBT and were incubated for 1 h with biotinylated rabbit anti-goat immunoglobulin (1:200; Vector Laboratories, Burlingame, CA) in the same solution. The sections were rinsed with PBT and were incubated for 1 h with avidin-biotin-peroxidase complex (Vector Laboratories) in PBT. Visualization of the immunohistochemical reaction was achieved by incubation with 3,3'-diaminobenzidine (DAB) with nickel-ammonium sulphate to which hydrogen peroxide was added (DAB kit, Vector Laboratories). After the staining procedure was completed, the sections were rinsed in PBS followed by distilled water and placed on slide glasses, which were dehydrated in 100% ethanol, and cleared in 100% xylene, and cover slips were placed.

Fluorescent Double Staining of c-Fos and Glutamic Acid Decarboxylase. Free-floating spinal cord sections were first incubated for 1 h in blocking solution consisting of 3% donkey serum (Chemicon International, Temecula, CA) in PBS. They were incubated overnight with goat anti–c-Fos antibody (1:1,000, catalog No. sc-52-G, Santa Cruz Biotechnology) and rabbit anti–glutamic acid decarboxylase (GAD) antibody (1:1,000, catalog No. GC3008, Affiniti Research Products, Mamhead, UK) in 1% donkey serum in PBS on a shaker at −4°C. Sections were rinsed with PBS and incubated for 1 h in

darkness with a mixture of Cy3-conjugated donkey anti-goat secondary antibody (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA) and fluorescein isothiocyanate (FITC)–conjugated donkey anti-rabbit secondary antibody (1:200, Jackson ImmunoResearch Laboratories) in 1% donkey serum in PBS. Sections were rinsed with PBS, floated in water, and mounted on slide glasses. After being dried in darkness, one drop of VectaShield (Vector Laboratories), a special mounting medium for fluorescence, was put on each slide glass, and a cover slip was placed.

Quantitation of c-Fos–positive Cells. Using a DAB staining, c-Fos–positive cells were identified by dense black nuclear staining under bright-field microscope (Olympus Model BX50 Research Photomicroscope; Olympus Optical, Southall, Middlesex, UK). Randomly selected sections were photographed using a digital camera (Olympus Digital Camera, model C2020Z, Olympus Optical). The number of c-Fos–positive cells was counted for each area of the spinal cord (*i.e.,* laminae I–II [superficial area], laminae III–IV [nucleus proprius area], laminae V–VI [neck area], and laminae VII–X [ventral area]), according to the method by Presley *et al.*,[16] based on the laminar scheme in rats originally developed by Molander *et al.*[17] Five photos were taken from each rat, and the mean number of c-Fos–positive cells per section was calculated. At least three to four animals were examined for each group, and the number of c-Fos–positive cells in each group was calculated as mean ± SD. The investigator was blinded to the treatment that the experimental groups had received.

Confocal Microscopic Analyses for Colocalization of c-Fos–positive and Glutamic Acid Decarboxylase–positive Cells. Colocalization of c-Fos and GAD staining was examined by confocal microscopy (Bio-Rad Microradiance MR/AG II System equipped with Argon/Green HeNe lasers; Bio-Rad Laboratories, Hercules, CA). Cy3 and FITC staining were detected separately with E570 LP (red) and HQ500 LP (green) emission filters, respectively.

Data Analysis. The numbers of c-Fos–positive cells were analyzed by one-way analysis of variance, and Bonferroni correction was used as a *post hoc* test. A P value < 0.05 was considered to be statistically significant.

Results

Time Course and Dose Response of Nitrous Oxide–induced c-Fos Expression in Dorsal Horn of Spinal Cord in Fischer Strain

The number of c-Fos–positive cells in the control group was approximately 40 cells/section, which was consistent among different experiments. The number of c-Fos–positive cells increased in a time-dependent manner after 75% N_2O exposure reaching to the peak level of approximately 130 cells/section at approximately 90 and 120 min (fig. 2). Increase in the number of c-Fos–positive cells after 90 min of nitrous oxide exposure was dose-dependent (fig. 3) and was observed in the entire area excluding laminae I and II (figs. 4 and 5), but appeared to be specifically concentrated in laminae III and IV (fig. 5).

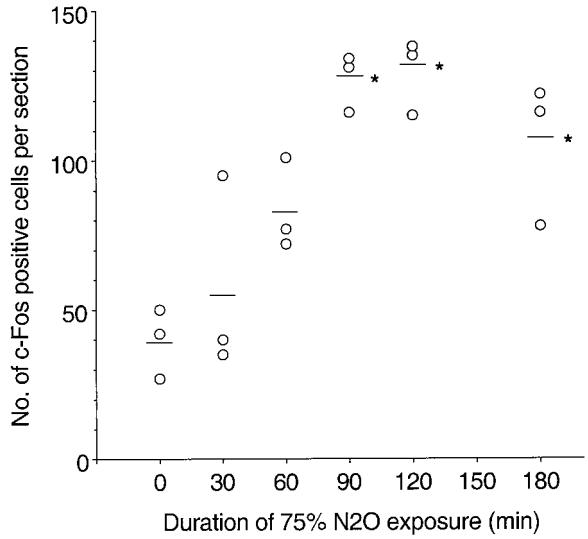

Fig. 2. Time course of 75% nitrous oxide (N_2O)–induced c-Fos expression in the spinal cord at the lumbar level (L5). Each closed circle represents the mean number of c-Fos–positive cells from five randomly selected sections from one animal. Each horizontal bar indicates the mean of three animals at each time point. *$P < 0.05$ compared with basal c-Fos expression (0 min, control).

Effect of Nitrous Oxide in Lewis Strain

The number of c-Fos–positive cells in the control group was 35.0 ± 15.7 cells/section (fig. 5). After 75% N_2O exposure for 90 min, the number of c-Fos–positive cells increased slightly in all laminae but statistical difference between the control groups was achieved only in laminae I and II (fig. 5).

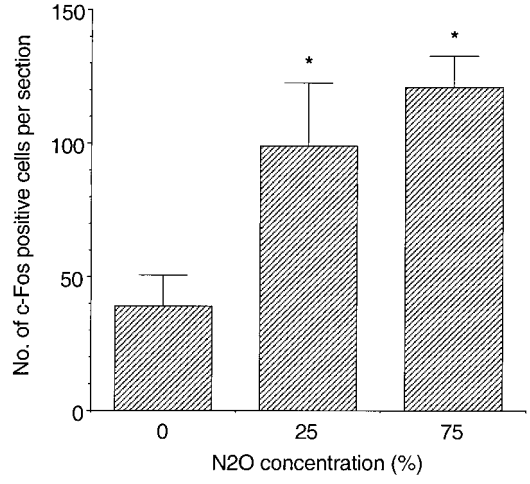

Fig. 3. The effect of nitrous oxide (N_2O) concentration (90-min exposure) on the total number of c-Fos–positive cells in the spinal cord at the lumbar level (L5). Each bar represents the mean number of c-Fos–positive cells from four animals, and the error bar indicates the SD. *$P < 0.05$ compared with basal c-Fos expression (0%, control).

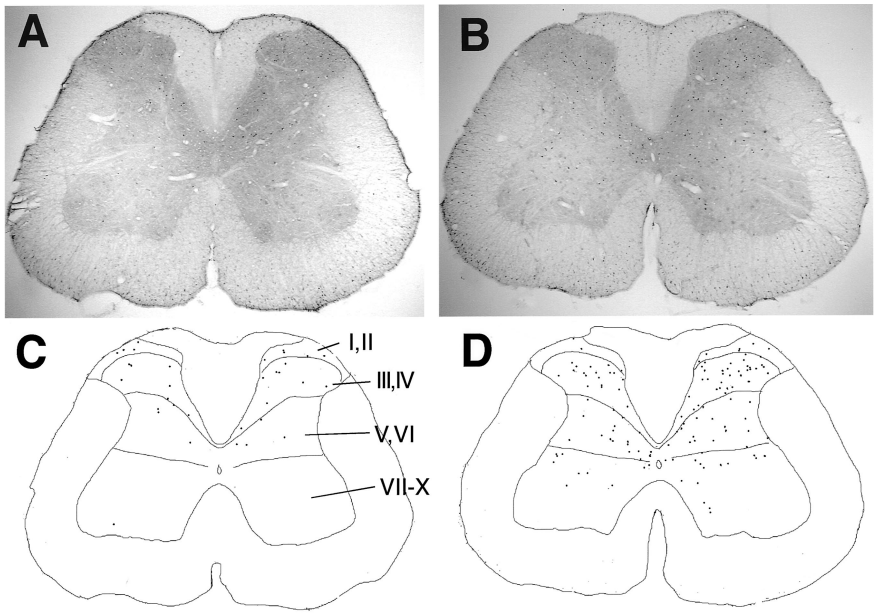

Fig. 4. (*A* and *B*) Representative cross-sections of the spinal cord at the lumbar level L5 stained for c-Fos using a 3,3'-diaminobenzidine (DAB) reaction and (*C* and *D*) illustrations of each section including the borders of each lamina. (*A* and *C*) Control (air for 90 min). (*B* and *D*) Seventy-five percent nitrous oxide (90 min). The c-Fos–positive cells are seen as block nuclear staining in *A* and *B* and are emphasized with large black dots in *C* and *D*.

Colocalization of c-Fos–positive Cells and GABAergic Neurons in Fischer Strain

Representative pictures of double staining for c-Fos and GAD are shown in figure 6. Compared with DAB staining for c-Fos alone, the number of c-Fos–positive cells in both control and nitrous oxide groups was lower in double-stained sections. The most well-stained undamaged section was selected from each animal, and all c-Fos–positive cells in half of each section were examined for colocalization with GAD. The prevalence of c-Fos and GAD colocalization was calculated for all the animals in each group (table 1). In the control group, a total of 53 c-Fos–positive cells were examined, and in 26 (49.1%) of them, GAD was colocalized. In the nitrous oxide group, a total of 216 cells were examined, and in 182 (84.3%) of them, GAD was colocalized. Because the number of c-Fos–positive cells that were not colocalized with GAD was almost the same between the control (27) and nitrous oxide (34) groups, these results indicate that almost all c-Fos–positive cells induced by nitrous oxide also contained GAD.

Discussion

c-Fos, a protein product of the immediate early gene, c-*fos*, has been commonly used as a histologic marker of transsynaptic neuronal activation since Hunt *et al.*[18] first

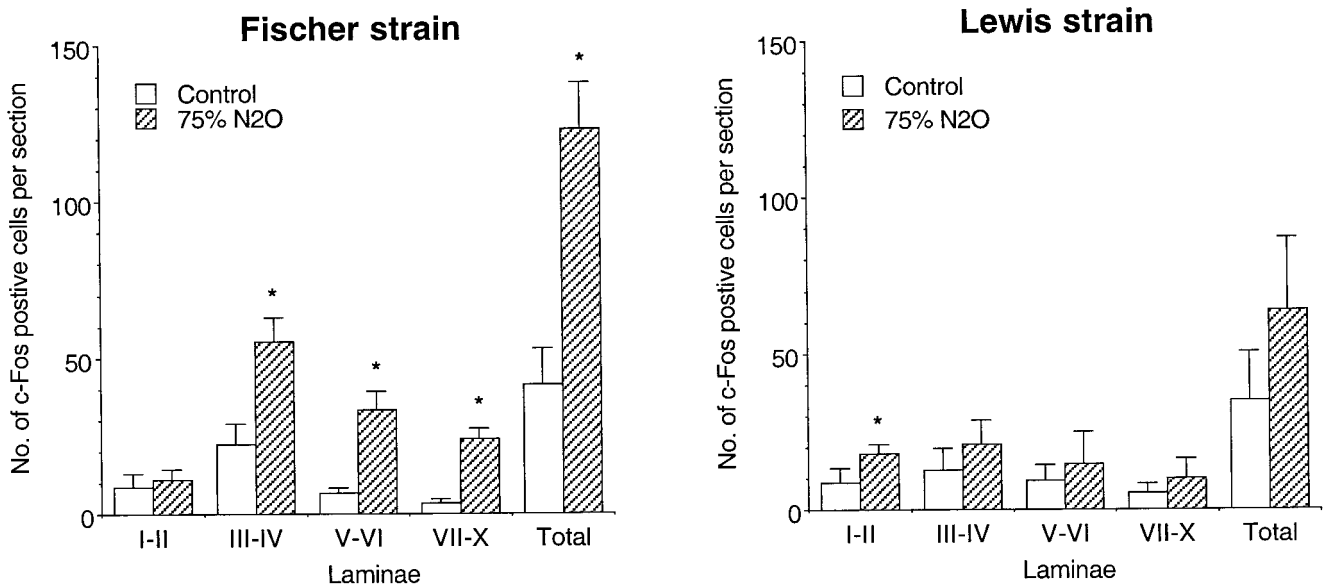

Fig. 5. The effect of nitrous oxide (75% N_2O for 2 h) on the total number of c-Fos–positive cells in each lamina of the spinal cord at the lumbar level L5 in Fischer and Lewis strains. Each bar represents the mean number of c-Fos–positive cells from four animals, and the error bar indicates the SD. *$P < 0.05$ compared with basal c-Fos expression (0%, control).

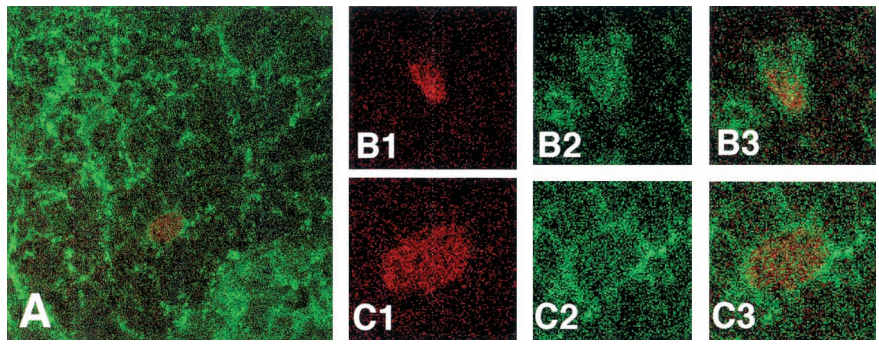

Fig. 6. Representative pictures of confocal microscopic analyses showing c-Fos–positive cells (red), glutamic acid decarboxylase–positive cells (green), and colocalization of c-Fos and glutamic acid decarboxylase within the same cell. (*A*) A lower magnification. (*B* and *C*) A higher magnification. The cell shown in *B1–3* is taken from laminar III, which is relatively small, and that in *C1–3* from laminar V, which is relatively large.

reported it in 1987. In the present study, we used c-Fos as a marker for neuronal pathways activated by nitrous oxide to examine whether GABAergic inhibitory interneurons are involved in the antinociceptive effect of nitrous oxide in the rat spinal cord. First, we characterized the expression pattern of c-Fos after nitrous oxide administration. Second, using double-staining techniques and confocal microscopic analyses, we demonstrated that the c-Fos–positive cells induced by nitrous oxide were GABAergic neurons. In Fischer strain, exposure to 75% N_2O increased the number of c-Fos–positive cells in the spinal cord approximately threefold beyond baseline expression. This effect was time-dependent (fig. 2) and dose-dependent (fig. 3). To the contrary, this effect was observed less prominently in the Lewis strain, which is consistent with a lack of antinociceptive effect by tail-flick test in this strain in the previous study of Fender *et al.*[15] In another recent study, our group has shown that nitrous oxide administration increased c-Fos expression in noradrenergic neurons of the brainstem (*i.e.,* A5, A6 [locus ceruleus], and A7) in Sprague-Dawley rats,[13] a strain in which nitrous oxide shows reasonably strong antinociceptive effect in the tail-flick test. Furthermore, when the rats were injected intracerebroventricularly with a mitochondrial toxin, saporin, which was coupled to the dopamine β hydroxylase antibody, those noradrenergic cells were almost entirely eliminated and the animals no longer showed the antinociceptive effect of nitrous oxide. Together with the results from the present study, our findings provide functional evidence for the involvement of descending noradrenegic neurons in the antinociceptive effect of nitrous oxide. In addition, we have found a striking colocalization of nitrous oxide–induced c-Fos expression with GABAergic neurons (table 1 and fig. 6), suggesting that GABAergic inhibitory interneurons and their downstream effectors are at least partly involved in the antinociceptive effect of nitrous oxide.

The distribution patterns of c-Fos–positive cells in the control and nitrous oxide groups of Fischer strain within the spinal cord are summarized in table 2 and juxtaposed with information regarding the localization of different neurons and receptors from previously published reports. In control animals that were exposed to air, the few c-Fos–positive cells were randomly distributed throughout all laminae. In the nitrous oxide group, the number of c-Fos–positive cells increased significantly, especially in laminae III–VI, and were almost entirely colocalized with GABAergic neurons (table 1 and fig. 6). Although GABAergic neurons are ubiquitous in the entire spinal cord (except for lamina IX),[19] they are most prevalent in laminae I–III, which are proposed to be inhibitory interneurons.[20] The noradrenergic descending neurons from the brainstem have been shown to

Table 1. The Number of Glutamic Acid Decarboxylase–Positive Cells Among c-Fos–Positive Cells in the Spinal Cord (L5) in Fischer Rats

	Animal				
	1	2	3	4	Total
Control					
Laminae I–II	0/1	0/2	0/1	1/2	1/6 (16.7%)
Laminae III–IV	4/7	2/4	6/10	5/10	17/31 (54.8%)
Laminae V–VI	1/2	1/2	4/6	2/2	8/12 (66.7%)
Laminae VII–X	0/1	0/1	0/1	0/1	0/4 (0.0%)
Total	5/11	3/9	10/18	8/15	26/53 (49.1%)
75% N_2O					
Laminae I–II	2/3	2/2	1/1	2/3	7/9 (77.8%)
Laminae III–IV	20/22	16/20	22/28	37/43	95/113 (84.1%)
Laminae V–VI	11/12	15/16	13/18	19/22	58/68 (85.3%)
Laminae VII–X	2/3	4/4	6/7	10/12	22/26 (84.6%)
Total	35/40	37/42	42/54	68/80	182/216 (84.3%)

The best stained section was selected from each animal, and all c-Fos–positive cells in one half of the section were examined by confocal microscope.

Table 2. The Distribution Pattern of c-Fos–Positive Cells in the Control and N_2O Groups in Comparison with Those of Various Neurons and Receptors

Lamina	I	II	III	IV	V	VI	VII–X
c-Fos, control	±	±	±	±	±	±	±
c-Fos, N_2O	±	±	++	++	++	++	+
GABAergic neurons[19]	++	++	++	+	+	+	+*
Noradrenergic descending neurons[21]	++	++	++	++			
Primary afferent neurons[24]	++	++			++		
α_2 Adrenoceptors[25,26]	++	++			+		
α_1 Adrenoceptors[27]	+	+	+	+	+	+	+

* Except for IX.

N_2O = nitrous oxide; GABAergic = γ-aminobutyric acid–mediated.

terminate in the spinal cord mainly in laminae I–IV,[21] although the distribution of the termini depends on the genetic background and its origin in the brainstem (*i.e.,* A5, A6 [locus ceruleus] or A7).[22,23] Based on our data, we suggest that nitrous oxide–induced c-Fos–positive cells, particularly in laminae III and IV, are activated by nitrous oxide through descending noradrenergic neurons, although this has not yet been tested definitively. Nitrous oxide– induced c-Fos–positive cells in other laminae could be the result of (1) the activation of a minority group of descending noradrenergic neurons that reach laminae other than I–IV, (2) activation of other descending neurons (*e.g.,* serotonergic or opioidergic neurons), or (3) direct activation by nitrous oxide in the spinal cord. Further investigations are necessary to determine their origins and whether the activation of these neurons is functionally involved in the antinociceptive effect of nitrous oxide. Interestingly, the distribution pattern of nitrous oxide–induced c-Fos–positive cells does not correspond with that of the primary afferent neurons, which are mainly in laminae I, II, and V,[24] or that of α_2 adrenoceptors, which mostly corresponds to that of the primary afferent neurons.[25,26] The α_1 adrenoceptors are localized throughout the spinal cord.[27] Because nitrous oxide administration did not increase the number of c-Fos–positive cells in laminae I and II in Fischer strain, descending noradrenergic neurons that terminate at these laminae may be acting in an inhibitory manner, possibly through α_2 adrenoceptors (any inhibitory action caused by nitrous oxide would not result in increasing c-Fos expression).

GABA usually produces postsynaptic inhibition by hyperpolarizing the postsynaptic cell (fig. 1). In support of this mechanism in the spinal cord, a recent electrophysiologic study has shown that norepinephrine applied in the perfusion solution of the sliced rat spinal cord preparation activates GABAergic inhibitory activity in the substantia gelatinosa of the spinal cord (*i.e.,* lamina II in the dorsal horn).[28] It also has been demonstrated that this effect of norepinephrine is mediated by α_1 but not by α_2 adrenoceptors.[28] GABA may also act as a depolarizing transmitter on the presynaptic terminals of the primary afferent neurons to produce presynaptic inhibition (fig. 1).[29] In an immunohistochemical study using anti-GABA antiserum, it has been shown that some unmyelinated primary afferent neurons are subject to presynaptic inhibition at GABAergic neurons.[30] Furthermore, supporting the existence of the latter function, gene expression of the $GABA_A$ receptor has been demonstrated in the dorsal root ganglia in addition to the dorsal horn.[31]

The hypothetical neuronal pathways shown in fig. 1 are not an exhaustive representation because both serotonergic and opioidergic inhibitory neurons also exist, although their functional importance in the antinociceptive effect of nitrous oxide is not clear. Yet, nitrous oxide increased c-Fos–positive cells not only in laminae III and IV, where noradrenergic descending inhibitory neurons are mainly distributed, but also in other laminae, suggesting the possibility that other pathways are activated, although these may not necessarily be involved in the antinociceptive effect of nitrous oxide. It is entirely possible that multiple pathways work in concert to produce the antinociceptive effect of nitrous oxide; although all may be needed, none is sufficient.

In summary, we have demonstrated in the present study that nitrous oxide activates GABAergic neurons in the dorsal horn of the rat spinal cord. Our finding suggests that GABAergic interneurons and their downstream effectors play at least a part in the antinociceptive action of nitrous oxide in the spinal cord.

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References

1. Maze M, Fujinaga M: Recent advances in understanding the actions and toxicity of nitrous oxide. Anaesthesia 2000; 55:311–4

2. Chapman CR, Benedetti C: Nitrous oxide effects on cerebral evoked potential to pain: Partial reversal with a narcotic antagonist. ANESTHESIOLOGY 1979; 51:135–8

3. Yang JC, Clark WC, Ngai SH: Antagonism of nitrous oxide analgesia by naloxone in man. ANESTHESIOLOGY 1980; 52:414–7

4. Berkowitz BA, Finck AD, Ngai SH: Nitrous oxide analgesia: Reversal by naloxane and development of tolerance. J Pharm Exp Ther 1977; 203:539–47

5. Hodges BL, Gagnon MJ, Gillespie TR, Breneisen JR, O'Leary DF, Hara S,

Quock RM: Antagonism of nitrous oxide antinociception in the rat hot plate test by site-specific mu and epsilon opioid receptor blockage. J Pharm Exp Ther 1994; 269:596–600

6. Guo T-Z, Davies MF, Kingery WS, Patterson AJ, Limbird LE, Maze M: Nitrous oxide produces antinociceptive response *via* α_{2B} and/or α_{2C} adrenoceptor subtypes in mice. ANESTHESIOLOGY 1999; 90:470–6

7. Quock RM, Graczak LM: Influence of narcotic antagonist drugs upon nitrous oxide analgesia in mice. Brain Res 1988; 440:35–41

8. Quock RM, Curtis BA, Reynolds BJ, Mueller JL: Dose-dependent antagonism and potentiation of nitrous oxide antinociception by naloxone in mice. J Pharm Exp Ther 1993; 267:117–22

9. Fang F, Guo TZ, Davies MF, Maze M: Opiate receptors in the periaqueductal gray mediate analgesic effect of nitrous oxide in rats. Eur J Pharm 1997; 336: 137–41

10. Guo T-Z, Poree L, Golden W, Stein J, Fujinaga M, Maze M: Antinociceptive response to nitrous oxide is mediated by supraspinal opiate and spinal α_2 adrenergic receptors in the rat. ANESTHESIOLOGY 1996; 85:846–52

11. Zuniga J, Joseph S, Knigge K: Nitrous oxide analgesia. partial antagonism by naloxone and total reversal after periaqueductal gray lesions in the rat. Eur J Pharm 1987; 142:51–60

12. Zhang C, Davies MF, Guo T-Z, Maze M: The analgesic action of nitrous oxide is dependent on the release of norepinephrine in the dorsal horn of the spinal cord. ANESTHESIOLOGY 1999; 91:1401–7

13. Sawamura S, Kingery WS, Davies MF, Agashe GS, Clark JD, Kobilka BK, Hashimoto T, Maze M: Antinociceptive action of nitrous oxide is mediated by stimulation of noradrenergic neurons in the brainstem and activation of α2B adrenoceptors. J Neurosci 2000; 20:9242–51

14. Komatsu T, Shingu K, Tomemori N, Urabe N, Mori K: Nitrous oxide activates the supraspinal pain inhibition system. Acta Anaesth Scand 1981; 25:519–22

15. Fender C, Fujinaga M, Maze M: Strain differences in antinociceptive effect of nitrous oxide on tail flick test in rats. Anesth Analg 2000; 90:195–9

16. Presley RW, Menétrey D, Levine JD, Basbaum AI: Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. J Neurosci 1990; 10:323–35

17. Molander C, Xu Q, Grant G: The cytoarchitectonic organization of the spina cord in the rat: I. The lower thoracic and lumbosacral cord. J Comp Neurol 1984; 230:133–41

18. Hunt SP, Pini A, Evan G: Induction of c-fos–like protein in spinal cord neurons following sensory stimulation. Nature 1987; 328:632–4

19. Barbar RP, Vaughn JE, Roberts E: The cytoarchitecture of GABAergic neurons in rat spinal cord. Brain Res 1982; 238:305–28

20. Todd AJ, Sullivan AC: Light microscopic study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. J Comp Neurol 1990; 296:496–505

21. Clark FM, Proudfit HK: The projection of noradrenergic neurons in the A7 catecholamine cell group to the spinal cord in the rat demonstrated by anterograde tracing combined with immunocytochemistry. Brain Res 1991; 547:279–88

22. Sulka KA, Westlund KN: Spinal projections of the locus coeruleus and the nucleus subcoeruleus in the Harlan and the Sasco Sprague-Dawley rat. Brain Res 1992; 579:67–73

23. Clark FM, Proudfit HK: Anatomical evidence for genetic differences in the innervation of the rat spinal cord by noradrenergic locus coeruleus neurons. Brain Res 1992; 591:44–53

24. Besson JM, Chaouch A: Peripheral and spinal mechanisms of nociception. Physiol Rev 1987; 67:67–186

25. Rosin DL, Zeng D, Stornetta RL, Norton FR, Riley T, Okusa MO, Guyenet PG, Lynch KR: Immunohistochemical localization of α2A-adrenergic receptors in catecholaminergic and other brainstem neurons in the rat. Neuroscience 1993; 56:139–55

26. Stone LS, Broberger C, Vulchanova L, Wilcox GL, Hökfelt T, Riedl MS, Elde R: Differential distribution of α2A and α2C adrenergic receptor immunoreactivity in the rat spinal cord. J Neurosci 1998; 18:5928–37

27. Pieribone VA, Nicholas AP, Dagerlind A, Hokfelt T: Distribution of alpha 1 adrenoceptors in rat brain revealed by in situ hybridization experiments utilizing subtype-specific probes. J Neurosci 1994; 14:4252–68

28. Baba H, Goldstein PA, Okamoto M, Kohno T, Ataka T, Yoshimura M, Shimoji K: Norepinephrine facilitates inhibitory transmission in substantia gelatinosa of adult rat spinal cord: II. Effects on somatodendritic sits of GABAergic neurons. ANESTHESIOLOGY 2000; 92:485–92

29. Barber RP, Vaughn JE, Saito K, McLaughlin BJ, Roberts E: GABAergic terminals are presynaptic to primary afferent terminals in the substantia gelatinosa of the rat spinal cord. Brain Res 1978; 141:35–55

30. Todd AJ, Lochhead V: GABA-like immunoreactivity in type I glomeruli of rat substantia gelatinosa. Brain Res 1990; 514:171–4

31. Persohn E, Malherbe P, Richards JG: In situ hybridization histochemistry reveals a diversity of GABAA receptor subunit mRNAs in neurons of the rat spinal cord and dorsal root ganglia. Neuroscience 1991; 42:497–507