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Evidence for the Involvement of Spinal Cord α_1 Adrenoceptors in Nitrous Oxide–induced Antinociceptive Effects in Fischer Rats

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Background: **In a previous study, the authors found that nitrous oxide (N_2O) exposure induces c-Fos (an immunohistochemical marker of neuronal activation) in spinal cord γ-aminobutyric acid–mediated (GABAergic) neurons in Fischer rats. In this study, the authors sought evidence for the involvement of α_1 adrenoceptors in the antinociceptive effect of N_2O and in activation of GABAergic neurons in the spinal cord.**

Methods: **Adult male Fischer rats were injected intraperitoneally with α_1 adrenoceptor antagonist, α_2 adrenoceptor antagonist, opioid receptor antagonist, or serotonin receptor antagonist and, 15 min later, were exposed to either air (control) or 75% N_2O. In some animals, nociception was investigated with the plantar test after 30 min of exposure, while in other animals, gas exposure was continued for 90 min and the spinal cord was examined for c-Fos immunostaining. In a separate experiment, animals were exposed to the above gases alone, after which the spinal cords were examined immunohistochemically for c-Fos and α_1 adrenoceptor by double-staining methods.**

Results: **The antinociceptive effect of N_2O was attenuated by prazosin (an α_1 adrenoceptor antagonist), yohimbine (an α_2 adrenoceptor antagonist), and naloxone (an opioid receptor antagonist) but not by methysergide and tropisetron (serotonin receptor antagonists). N_2O exposure induced c-Fos expression in the spinal cord, which was blocked by prazosin and naloxone but not by other drugs. N_2O-induced c-Fos expression was colocalized with α_1 adrenoceptor immunoreactivity in laminae III–IV.**

Conclusions: **These findings support the hypothesis that N_2O activates GABAergic interneurons through α_1 adrenoceptors to produce its antinociceptive effect.**

WE have sought to characterize the molecular mechanism and neural substrates involved in the antinociceptive action of nitrous oxide (N_2O). In brief, N_2O induces opioid peptide release in the brain stem, leading to the activation of the descending noradrenergic inhibitory neurons, which results in modulation of the pain–nociceptive processing in the spinal cord.[1] Available evidence suggests that at the level of the spinal cord, there appear to be at least two neuronal systems that are involved (fig. 1). In one of the pathways, activation of the α_2 adrenoceptors produces either direct presynaptic inhibition of neurotransmitter release from primary afferent neurons or postsynaptic inhibition of the second-order neurons.[1] In a second hypothetical pathway, we propose that inhibitory γ-aminobutyric acid–mediated (GABAergic) interneurons are activated *via* α_1 adrenoceptors, resulting in either presynaptic inhibition of the nociceptive primary afferent neurons or postsynaptic inhibition of second-order neurons.[2] In this study, we sought evidence to link the participation of GABAergic neurons in the antinociceptive effect of N_2O to their activation by α_1 adrenoceptors.

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Materials and Methods

Animals

Adult male Fischer rats (11–12 weeks old) were used throughout the study (B&K Universal, Grimston Aldbrough, Hull, United Kingdom). All animal procedures were carried out in accordance with the United Kingdom (Scientific Procedures) Act of 1986, and the study protocol was approved by the Home Office of the United Kingdom (London, United Kingdom). All efforts were made to minimize animal suffering and reduce the number of animals used.

Drug Treatment

Animals were injected intraperitoneally with the following drugs 15 min before gas exposure: prazosin, an α_1 adrenoceptor antagonist (Cat. No. 0623; Tocris Cookson, Ballwin, MO); yohimbine, an α_2 adrenoceptor antagonist (Cat. No. Y-3125; Sigma Chemical Co., St Louis, MO); naloxone, an opioid receptor antagonist (Cat. No. N-7758; Sigma Chemical Co.); methysergide, a nonselective 5-HT receptor antagonist (Cat. No. 1064; Tocris Cookson); and tropisetron, a selective 5-HT3 receptor antagonist (Cat. No. T-104; Sigma Chemical Co.). Dosages of each drug examined were derived from the literature.[3–6] All drugs were dissolved in saline except

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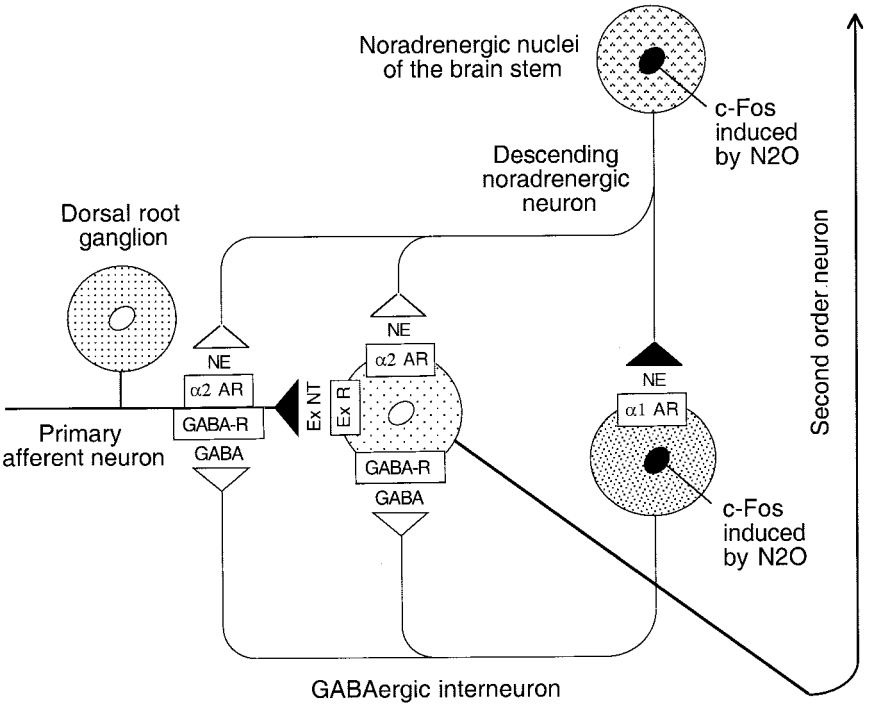

Fig. 1. Putative neuronal pathways in the spinal cord involved in the antinociceptive effects of N_2O. Closed triangles indicate excitatory synapses, and open triangles indicate inhibitory synapses. Small closed circles indicate the nucleus of cells activated by N_2O exposure, and a small open circle indicates the nucleus of a cell inactivated by N_2O exposure. There are at least two neuronal systems that may be involved: (1) direct presynaptic inhibition of the nociceptive primary afferent neurons and/or postsynaptic inhibition of the second-order neurons through activation of the α_2 adrenoceptors, and (2) indirect presynaptic inhibition of the nociceptive primary afferent neurons and/or postsynaptic inhibition of the second-order neurons by the activation of GABAergic inhibitory interneurons through α_1 adrenoceptors. α_2 AR = α_2 adrenoceptor; Ex NT = excitatory neurotransmitters; Ex-R = receptors for excitatory neurotransmitters; GABA = γ-aminobutyric acid; GBA-R = $GABA_A$ receptor; NE = norepinephrine.

for prazosin, which was dissolved in heated distilled water. The injection volume was standardized as 1 ml.

Gas Exposure

Gas exposure was performed in an acryl plastic exposure chamber (18 in long, 9 in wide, and 8 in high). Either a mixture of 75% N_2O and 25% O_2 or air at a flow rate of 4 l/min was continuously delivered into the exposure chamber *via* an inflow port and exhausted *via* an outflow port. Gas concentrations, including those for N_2O, O_2, and CO_2, in the chamber were measured continuously by infrared gas spectrometry (Ohmeda 5250 RGM; Ohmeda, Hatfield, Hertz, United Kingdom). Animals were placed into the chamber through the side door after the desired gas concentrations were achieved and stabilized.

Plantar Test

One hour before the experiment (baseline) and 30 min after the initiation of gas exposure (which coincides with the peak antinociceptive effect of N_2O),[7] thermal nociceptive testing was performed using a plantar test device (Plantar test 7370; Ugo Basile, Comerio, Italy). Radiant heat was applied on the plantar surface of hind paws through the floor of the exposure chamber, and the paw withdrawal latency (PWL), defined as the time between the activation of the heat source and hind-paw withdrawal, was automatically recorded. Heat intensity was adjusted such that the baseline PWL was approximately 4 s. To avoid tissue damage, a predetermined cutoff time of 10 s was imposed. Each PWL data set consisted of a mean of three trials for each animal. From the PWL, the percentage of maximal possible effect (%MPE) was calculated as follows:

$$\%MPE = \{(PWL \text{ with treatment} - \text{Baseline PWL})/(\text{Cutoff time} - \text{Baseline PWL})\} \times 100$$

Spinal Cord Preparation and Cryosection

In another set of experiments, animals were not subjected to nociceptive testing but were killed by an overdose of sodium pentobarbital (100 mg/kg), intraperitoneal, following 90 min of gas exposure, which is the time to the peak effect of c-Fos induction in the spinal cord after N_2O exposure.[2] During the terminal anesthetic, the animals were perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer *via* a 16-gauge cannula inserted through the left ventricle into the ascending aorta. Following decapitation, the spinal cord was expelled by rapid injection of PBS at the sacral vertebral level and stored in 30% sucrose in 0.1 M phosphate buffer for at least 24 h at 4°C. A 5-mm portion of the spinal cord at the lumbar enlargement was cut by a razor blade and was freeze-mounted in embedding matrix, and 30-μm transverse sections were cut at −15°C; every third section was collected in PBS (approximately 40–50 sections per sample).

Immunohistochemistry: Diaminobenzidine Staining of c-Fos

Approximately 15–20 undamaged free-floating spinal cord sections were selected and were first incubated at

room temperature for 30 min in 0.3% hydrogen peroxide in 70% methanol–PBS and for 1 h in blocking solution consisting of 3% rabbit serum and 0.3% Triton X in PBS (PBT), followed by overnight incubation with goat anti–c-Fos antibody (1:10,000, Cat. No. sc-52-G; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution (1% normal rabbit serum in PBS) on a shaker at 4°C. Sections were then rinsed with PBT, incubated for 1 h with biotinylated rabbit antigoat immunoglobulin (1:200; Vector Laboratories, Burlingame, CA) in the same solution, rinsed with PBT, and incubated for 1 h with avidin–biotin–peroxidase complex (Vector Laboratories) in PBT. Visualization of the immunohistochemical reaction was achieved by incubation with DAB with nickel–ammonium sulfate (DAB kit; Vector Laboratories). After the staining procedure was completed, sections were rinsed in PBS followed by distilled water, mounted on slide glasses that were dehydrated in 100% ethanol, and cleared in 100% xylene, and cover slips were applied.

Quantitation of c-Fos–positive Cells

Using a DAB staining with nickel enhancement, c-Fos–positive cells were identified by dense black nuclear staining under a bright field microscope (Olympus Model BX50 Research Photomicroscope; Olympus Optical, Southall, Middlesex, United Kingdom). Five randomly selected, undamaged sections from each rat 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.*[8] Each group was comprised of at least four animals, and the number of c-Fos–positive cells in each group was calculated as mean ± SD. The investigator was blinded to the treatment cohort.

Immunohistochemistry: Fluorescent Double Staining of c-Fos and α_1 Adrenoceptor

In some animals, the spinal cord was collected after 90 min of gas exposure (either air or 75% N_2O) without drug pretreatment or nociceptive testing. Approximately 6–8 undamaged free-floating spinal cord sections from each specimen were first incubated for 1 h in blocking solution consisting of 3% donkey serum (Chemicon International, Temecula, CA) in PBS. They were then incubated overnight with goat anti–c-Fos antibody (1:1,000, Cat. No. sc-52-G; Santa Cruz Biotechnology) and rabbit anti–α_1 adrenoceptor antibody (1:1,000, Cat. No. PC160; Oncogene Research Products, Cambridge, United Kingdom) in 1% donkey serum in PBS on a shaker at 4°C. Sections were rinsed with PBT, incubated for 1 h in darkness with a mixture of Cy3-conjugated donkey antigoat secondary antibody (1:200; Jackson Immuno Research Laboratories, West Grove, PA) and FITC-conjugated donkey antirabbit secondary antibody (1:200; Jackson Immuno Research Laboratories) in 1% donkey serum in PBS, then rinsed with PBS, floated in water, and mounted on slide glasses. After being dried in darkness, cover slips were applied to the slides with one drop of VectaShield (Vector Laboratories) mounting medium for fluorescence. The best-preserved undamaged section was selected from each animal for analysis. In each laminal scheme, all c-Fos–positive cells were examined for colocalization with α_1 adrenoceptors under a fluorescent microscope by the investigator who was blinded to the treatment cohort (Leica DMR microscope; Leica, Wetzlar, Germany). Results from four animals for each group were summed, and the prevalence of c-Fos–α_1 adrenoceptor colocalization was calculated.

Data Analysis

Results from the plantar test, *i.e.*, %MPE, were compared for each drug treatment within the following groups; air–saline, air–drug, 75% N_2O–saline, and 75% N_2O–drug. The data were analyzed by one-way analysis of variance, and the Dunn test was used as an *a posteriori* test. Results from c-Fos single staining were compared in the same way for the entire spinal cord section and for each laminal scheme. Results from c-Fos and α_1 adrenoceptor double staining were compared between the air and 75% N_2O groups in each laminal scheme or total of the spinal cord. Data were analyzed using the Fisher exact test. In addition, the number of c-Fos–positive cells among either α_1 adrenoceptor–positive or –negative cells was compared between air and 75% N_2O groups by one-way analysis of variance. A P value less than 0.05 was considered to be statistically significant.

Results

Plantar Test

The animals exposed to air were awake and active during the experiment, while those animals exposed to N_2O were excited for the first 5–10 min of exposure, followed by a relatively calm state. The animals injected with prazosin became deeply sedated after N_2O exposure, but other drugs did not have this effect. The results from the plantar test are summarized in table 1. The baseline reaction time was approximately 4.0 s in each group. Exposure to 75% N_2O increased the reaction time to 6.3 ± 0.4 s, or 36.8 ± 8.3% of MPE. None of the tested drugs alone showed any effect on reaction time. Prazosin, yohimbine, and naloxone almost completely blocked the N_2O-induced antinociceptive effect, *i.e.*, the reaction time was no different from the baseline value. Methysergide or tropisetron showed no effect on N_2O-induced antinociceptive effect.

Table 1. Effects of Various Receptor Antagonists on N_2O-induced Antinociceptive Effect by the Plantar Test

Exposure	Pretreatment (i.p.) Drug	Dose	No. of Animals Examined	Reaction time (s, Mean ± SD) Baseline	30 min	% MPE
Air	Saline		6	3.9 ± 0.2	3.8 ± 0.2	−1.2 ± 3.8
	Prazosin	1 mg/kg	6	3.8 ± 0.4	3.7 ± 0.4	−1.9 ± 8.7
	Yohimbine	1 mg/kg	6	3.8 ± 0.3	3.6 ± 0.2	−2.2 ± 5.3
	Naloxone	1 mg/kg	6	4.1 ± 0.4	4.0 ± 0.3	−1.1 ± 5.9
	Methysergide	1 mg/kg	6	4.0 ± 0.3	4.0 ± 0.2	−2.5 ± 9.8
		10 mg/kg	6	3.8 ± 0.4	3.9 ± 0.2	2.4 ± 6.3
	Tropisetron	1 mg/kg	6	4.2 ± 0.5	4.1 ± 0.5	−1.6 ± 6.0
75% N_2O	Saline		7	4.1 ± 0.2	6.3 ± 0.4	36.8 ± 8.3†
	Prazosin	1 mg/kg	6	4.3 ± 0.4	4.1 ± 0.5	−4.8 ± 11.4*
	Yohimbine	1 mg/kg	6	3.9 ± 0.3	3.6 ± 0.3	−4.7 ± 6.0*
	Naloxone	1 mg/kg	6	4.4 ± 0.2	4.8 ± 0.6	7.0 ± 9.9*
	Methysergide	1 mg/kg	6	4.1 ± 0.4	6.0 ± 0.6	36.1 ± 8.6†
		10 mg/kg	6	4.0 ± 0.6	6.6 ± 0.7	41.9 ± 11.9†
	Tropisetron	1 mg/kg	6	3.8 ± 0.2	6.2 ± 0.3	38.4 ± 4.2†

* $P < 0.05$ *vs.* 75% N_2O/saline group among N_2O exposed groups; † $P < 0.05$ *vs.* air/saline group.
% MPE = percent of maximum possible effect; i.p. = intraperitoneal injection.

Nitrous Oxide–induced c-Fos Expression in the Spinal Cord

Results from the c-Fos staining experiments are summarized in table 2. The number of c-Fos–positive cells in the entire area of the spinal cord section in the air–saline group was 69.0 ± 7.9 (mean ± SD). Exposure to 75% N_2O increased the number of c-Fos–positive cells approximately twofold, to 142.8 ± 5.2. An increase in c-Fos–positive cells was observed in laminae III–IV, V–VI, and VII–X but not in laminae I–II. None of the tested drugs alone showed an effect on the number of c-Fos–positive cells compared with that of the air–saline group. Prazosin and naloxone significantly reduced the total number of c-Fos–positive cells in the spinal cord when compared with the N_2O–saline group. Prazosin nearly completely blocked the c-Fos expression in laminae III–IV (32.2 ± 2.1 *vs.* air–saline, 29.0 ± 3.7), but the effect of naloxone in laminae III–IV was only partial (1 mg/kg, 56.2 ± 2.8; 10 mg/kg, 43.5 ± 2.9). Neither drug had an inhibitory effect on c-Fos expression in laminae V–VI and VII–X. Yohimbine, methysergide, and tropisetron had no effect on N_2O-induced c-Fos expression in any lamina.

Colocalization of c-Fos–positive Cells and α_1 Adrenoceptors in the Spinal Cord

In the control group, 52 (41.6%) of 125 c-Fos–positive cells in four animals examined were colocalized with α_1 adrenoceptors. In the N_2O group, 119 (50.0%) of 238 cells in four animals examined showed c-Fos colocalization with α_1 adrenoceptors. Statistical differences were obtained between the two groups for those in laminae III–IV (table 3). When the results were analyzed separately in α_1 adrenoceptor–positive and –negative cells

Table 2. Effects of Various Receptor Antagonists on the Number of c-Fos Positive Cells in the Lumbar Spinal Cord (Mean ± SD)

Exposure	Pretreatment (i.p.) Drug	Dose	No. of Animals Examined	No. of Cells in Entire Section	No. of Cells Per Section I–II	III–IV	V–VI	VII–X
Air	Saline		4	69.0 ± 7.9	9.0 ± 2.2	29.0 ± 3.7	17.8 ± 3.6	13.2 ± 1.0
	Prazosin	1 mg/kg	4	72.8 ± 7.4	11.2 ± 0.5	26.8 ± 2.5	19.8 ± 4.0	15.0 ± 4.2
	Yohimbine	1 mg/kg	4	71.8 ± 6.2	8.0 ± 2.8	27.8 ± 3.9	21.2 ± 1.5	14.8 ± 2.2
		10 mg/kg	4	72.8 ± 4.3	7.8 ± 2.2	33.8 ± 3.6	19.8 ± 3.8	11.5 ± 3.0
	Naloxone	10 mg/kg	4	70.8 ± 10.7	8.0 ± 2.2	29.5 ± 3.9	20.2 ± 3.3	13.0 ± 2.4
	Methysergide	10 mg/kg	4	72.0 ± 5.4	6.2 ± 1.5	30.8 ± 3.1	20.2 ± 1.3	14.8 ± 1.0
	Tropisetron	1 mg/kg	4	68.5 ± 3.1	6.2 ± 1.0	29.2 ± 2.2	19.8 ± 1.3	13.5 ± 3.7
75% N_2O	Saline		4	142.8 ± 5.2†	12.2 ± 2.1	73.5 ± 2.4†	32.2 ± 3.3†	24.8 ± 4.5†
	Prazosin	1 mg/kg	4	93.8 ± 10.2†*	10.8 ± 1.5	32.2 ± 2.1*	29.5 ± 4.2†	21.2 ± 4.9
	Yohimbine	1 mg/kg	4	141.0 ± 10.4†	13.5 ± 3.5	76.2 ± 5.8†	30.0 ± 3.4†	21.2 ± 5.3†
		10 mg/kg	4	142.5 ± 7.0†	12.0 ± 0.8	77.2 ± 4.6†	32.2 ± 3.8†	21.0 ± 1.6†
	Naloxone	1 mg/kg	4	129.0 ± 7.7†	10.5 ± 2.5	56.2 ± 2.8†*	37.2 ± 2.6†	25.0 ± 2.9†
		10 mg/kg	4	106.2 ± 5.0†*	8.2 ± 0.5	43.5 ± 2.9†*	31.2 ± 5.7†	23.2 ± 2.8†
	Methysergide	1 mg/kg	4	136.2 ± 5.3†	9.0 ± 1.4	72.5 ± 4.7†	34.0 ± 2.4†	20.8 ± 3.6†
		10 mg/kg	4	130.8 ± 9.5†	8.5 ± 1.0	69.0 ± 2.9†	32.8 ± 4.2†	20.5 ± 3.1†
	Tropisetron	1 mg/kg	4	141.0 ± 7.8†	10.2 ± 1.0	73.5 ± 2.6†	34.2 ± 3.7†	23.0 ± 3.6†

* $P < 0.05$ *vs.* 75% N_2O/saline group among N_2O exposed groups; † $P < 0.05$ *vs.* air/saline group.
i.p. = intraperitoneal injection.

Table 3. The Number of α_1 Adrenoceptor Positive Cells among c-Fos Positive Cells in the Lumbar Spinal Cord

	Animal				
	1	2	3	4	Total
Air					
Laminae I–II	1/4	0/3	0/2	1/2	2/11 (18.1%)
Laminae III–IV	7/15	6/14	5/12	4/13	22/54 (40.7%)
Laminae V–VI	4/9	5/10	5/9	3/8	17/36 (47.2%)
Laminae VII–X	3/6	4/7	2/6	2/5	11/24 (45.8%)
Total	15/34	15/34	12/29	10/28	52/125 (41.6%)
75% N_2O					
Laminae I–II	1/4	1/5	0/3	0/4	2/16 (12.5%)
Laminae III–IV	24/31	19/28	18/30	20/34	81/123 (65.9%)
Laminae V–VI	5/15	6/18	6/17	7/17	24/67 (35.8%)
Laminae VII–X	4/12	2/5	3/7	3/8	12/32 (37.5%)
Total	34/62	28/56	27/57	30/63	119/238 (50.0%)

* $P < 0.05$ *vs.* air.

(fig. 2), N_2O induced c-Fos expression in α_1 adrenoceptor–positive cells in laminae III–IV and V–VI, and also in α_1 adrenoceptor–negative cells in laminae V–VI. Representative pictures of double staining for c-Fos and α_1 adrenoceptors in laminae III–IV are shown in figure 3 (a color version of this figure is available on the ANESTHESIOLOGY Web site at http://anesthesiology.org).

Discussion

The primary aim of the current study was to investigate whether α_1 adrenoceptors are involved in mediation of N_2O-induced antinociceptive effect and activation of GABAergic neurons in the spinal cord. We have shown that systemic administration of the α_1 adrenoceptor antagonist prazosin blocks N_2O-induced antinociceptive effect as measured by the plantar test (table 1) and inhibits N_2O-induced c-Fos expression in the spinal cord (table 2). In addition, double-staining analysis revealed that N_2O-induced c-Fos expression in laminae III–IV is strongly colocalized with α_1 adrenoceptors (table 3 and fig. 2). Apart from the caveats that prazosin was administered systemically, rather than intrathecally, and that a single dose was used, these data support our hypothetical "second" pathway mediating N_2O-induced antinociceptive effect in the spinal cord, *i.e.*, through the activation of inhibitory GABAergic interneurons *via* α_1 adrenoceptors (fig. 1). A previous report in mice in which prazosin blocked the antinociceptive effect of N_2O as measured by the tail-flick test in 129/svj strain is consistent with this pathway.[9] Further, a recent electrophysiological study demonstrated that norepinephrine applied to the sliced rat spinal cord preparation activates GABAergic inhibitory activity through α_1 but not α_2 adrenoceptors.[10]

Nitrous oxide exposure induced c-Fos expression in the spinal cord in most laminae except for laminae I–II, which is consistent with the findings from our previous

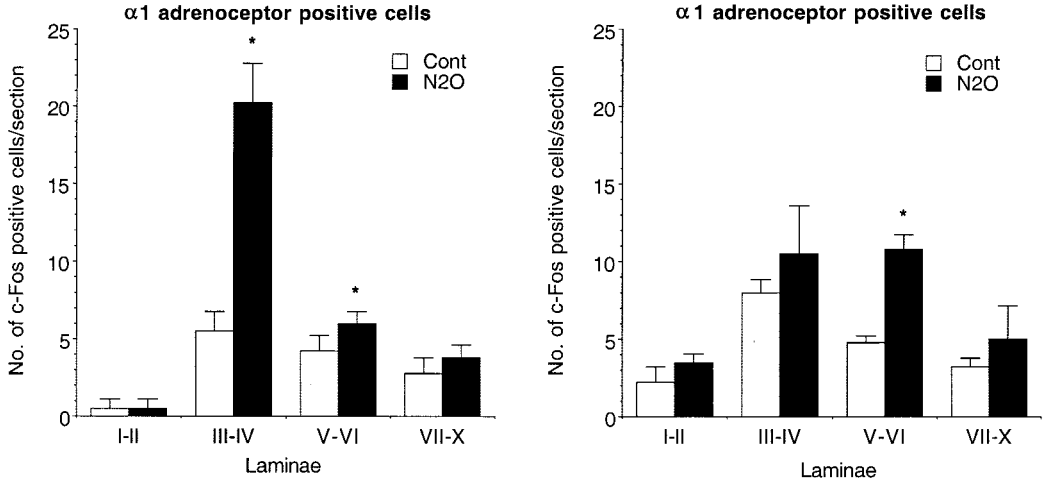

Fig. 2. The effect of 75% N_2O on the number of c-Fos–positive cells (mean ± SD) in each laminae of the spinal cord at the lumbar level in α_1 adrenoceptor–positive and –negative cells; analysis of the data in table 3 that are based on a total of eight animals. Open column indicates the number of c-Fos–positive cells in the air-exposed group (control). Closed column indicates the number of c-Fos–positive cells in the N_2O-exposed group. *$P < 0.05$ *versus* control.

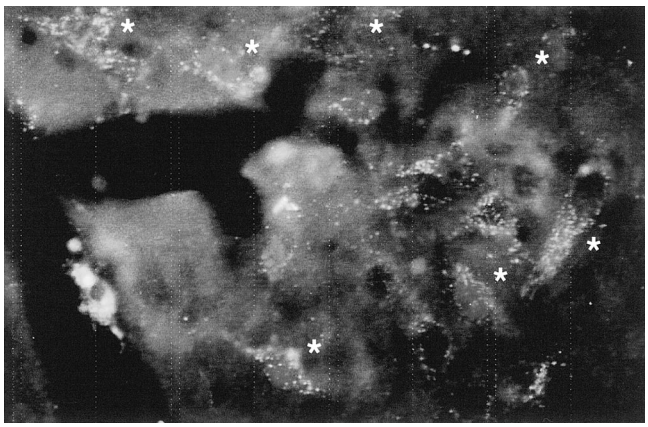

Fig. 3. Representative picture of the cells in laminae III–IV of lumbar level spinal cord double-stained for c-Fos (nuclear staining) and α_1 adrenoceptor (granular cellular staining). Those cells showing colocalization are indicated by asterisks.

study.[2] When the results from the double staining were analyzed in each lamina, the cells that expressed c-Fos during N_2O exposure showed the highest degree of colocalization with α_1 adrenoceptors in laminae III–IV (fig. 2). It is known that descending noradrenergic inhibitory neurons from the brain stem terminate in the spinal cord mainly in laminae I–IV,[11] while the distribution of the termini depends on the genetic background of the strain and the origin of the pathway in the brain stem, *i.e.*, A5, A6 (locus ceruleus), or A7.[12,13] The majority of the cells that expressed c-Fos induced by N_2O exposure in laminae V–VI (to some degree in laminae VII–X, as well) were not colocalized with α_1 adrenoceptors (fig. 2). Although prazosin inhibited N_2O-induced c-Fos expression in laminae III–IV, neither prazosin nor other receptor antagonists showed an inhibitory effect on c-Fos expression in laminae V–VI (table 2). In our previous study, we found that nearly all cells that express N_2O-induced c-Fos were GABAergic neurons.[2] Thus, N_2O-induced c-Fos–positive cells in laminae V–VI must be GABAergic neurons, but they are activated through receptors other than α_1 adrenoceptors, serotonin receptors, or opioid receptors. Further investigations are necessary to determine the identity of such receptors, although they may not be involved in the antinociceptive effect of N_2O.

We also examined the effects of other receptor antagonists on N_2O-induced antinociceptive effect, as measured by the plantar test, and c-Fos expression in the spinal cord. Yohimbine, an α_2 adrenoceptor antagonist, blocked N_2O-induced antinociceptive effects, a result in agreement with two previous studies.[3,14] Ohara *et al.*[14] reported that intraperitoneal injection of yohimbine (crosses the blood–brain barrier) but not L659–066 (an α_2 adrenoceptor antagonist that does not cross the blood–brain barrier) almost completely blocked the antinociceptive effects of N_2O on the tail-flick test in Sprague-Dawley rats. Guo *et al.*[3] reported that administration of the α_2 adrenoceptor antagonists (atipamezole, yohimbine, or N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) intrathecally but not intracerebroventricularly blocked the antinociceptive effects of N_2O on the tail-flick test in Sprague-Dawley rats, indicating that the site of antinociceptive action of α_2 adrenoceptor antagonists is at the spinal cord level. The result that yohimbine did not block N_2O-induced c-Fos expression in the spinal cord is also consistent with hypothesized antinociceptive pathways (fig. 1). Because activation of α_1 and α_2 adrenoceptors generally mediates excitatory and inhibitory neurotransmission, respectively, cells that express N_2O-induced c-Fos are activated *via* α_1 adrenoceptors and not by α_2 adrenoceptors. Thus, α_2 adrenoceptor antagonists would not be expected to affect N_2O-induced c-Fos expression in the spinal cord.

Berkowitz *et al.*[15] were the first to report on the inhibitory effects of opioid receptor antagonists against N_2O-induced antinociception in 1976. Since then, many investigators have reported similar inhibitory effects on N_2O-induced antinociceptive effect in other experimental paradigms and species, *e.g.*, in rats,[3,16–21] but some have reported that opiate receptor antagonists show no effect on N_2O-induced antinociception in humans[22–24] or in rats.[25,26] Gillman[27] considered that these negative reports are due in part to the inappropriate administration of naloxone and lack of consideration of naloxone's rapid decay in the brain after systemic administration. Opioid receptor antagonists appear to act at the supraspinal sites because intrathecal administration of opioid receptor antagonist does not block N_2O-induced antinociceptive effects in rats.[3]

In the current study, systemically administered naloxone almost completely blocked N_2O-induced antinociceptive effects (table 1), while the inhibitory effect of naloxone against N_2O-induced c-Fos expression was only partial (table 2). The reason for this discrepancy is unclear but may be explained by difference in timing of examination after naloxone injection. The plantar test was performed 45 min after administration, whereas the effect on c-Fos was examined 105 min after administration. For c-Fos experiments, we collected the spinal cord after 105 min of naloxone administration because it takes 60–90 min for c-Fos (protein) to be induced after N_2O exposure.[2] It does not necessarily mean that opioid receptors are needed to be blocked by naloxone during the entire period to attenuate N_2O-induced c-Fos expression, but we do not know the exact length of time required.

In addition to noradrenergic and opioidergic neurons, serotonergic neurons also play important roles in the descending inhibitory pain suppression system.[28] In this study, we examined two kinds of serotonin receptor antagonists, methysergide (nonselective 5-HT receptor antagonist) and tropisetron (selective 5-HT3 receptor antagonist), and found that neither blocked the antinociceptive effect

of N_2O or N_2O-induced c-Fos expression in the spinal cord. Most descending serotonergic inhibitory neurons originate from serotonergic nuclei in the medulla, *e.g.*, nucleus raphe magnus and the adjacent reticular formation. In a separate study, we recently demonstrated that N_2O exposure does not activate serotonergic nuclei in the medulla in Fischer rats, using a double staining analysis for c-Fos and tryptamine hydroxylase, a serotonin synthesizing enzyme.[29] However, one report contradicts this, indicating that the 5-HT3 receptor antagonist, ICS-205930, blocked the antinociceptive effects of N_2O as measured by the abdominal constriction test in Swiss-Webster mice, while the 5-HT1C/5-HT2 receptor antagonist, mianserin, potentiated this effect.[30] This controversy could be attributed to species or experimental paradigm differences, but further investigation is needed for clarification.

Exposure to 75% N_2O alone did not cause a hypnotic effect in rats; rather, initial exposure produced excitation. Interestingly, the combination of prazosin and N_2O caused a profound hypnotic effect, which was not observed in other treatment groups. Recent studies have suggested that activation of noradrenergic neurons in the locus ceruleus inhibits inhibitory GABAergic and galaninergic neurons in the ventrolateral preoptic nucleus, which results in activation (disinhibition) of histaminergic neurons in the tuberomammillary nucleus, which releases histamine into the cortex to promote arousal.[31–33] This neuronal pathway mediates the hypnotic effect of the α_2 adrenoceptor agonist dexmedetomidine when microinjected into the locus ceruleus, where it inhibits noradrenergic neurons through α_2 adrenoceptor activation.[34,35] In addition, a recent study in rats has shown that activation of α_1 adrenoceptors in the locus ceruleus suppresses the G-protein–coupled inward rectifier potassium (GIRK) conductance induced by α_2 adrenoceptor or μ-opioid receptor agonists.[36] Administration of 75% N_2O to the rats results in activation of noradrenergic neurons in the locus ceruleus[29,37] and excitation (arousal) rather than hypnosis. In light of the above, we propose that N_2O activates noradrenergic neurons that project to the locus ceruleus, which contain both α_1 and α_2 adrenoceptors; in aggregate, the effect of α_1 adrenoceptors exceeds that of α_2 adrenoceptors, resulting in suppression of GIRK and activation of the locus ceruleus. When the effect mediated by α_1 adrenoceptors is blocked by prazosin, the action mediated by α_2 adrenoceptors on GIRK predominates, which results in a hypnotic response.

In summary, we have demonstrated that systemic administration of the α_1 adrenoceptor antagonist prazosin blocks N_2O-induced antinociceptive effect, as measured by the plantar test, and inhibits N_2O-induced c-Fos expression in the spinal cord. Furthermore, double-staining analysis has revealed that N_2O-induced c-Fos expression is strongly localized in the cells in laminae III–IV with α_1 adrenoceptor immunoreactivity. These findings support our hypothesis that N_2O-induced antinociceptive effect is mediated by indirect inhibition of the nociceptive primary afferent neurons and/or postsynaptic inhibition of the second-order neurons by the activation of inhibitory GABAergic interneurons through α_1 adrenoceptors. In addition, we confirmed previous reports that the α_2 adrenoceptor antagonist yohimbine also blocks N_2O-induced antinociception, which also agrees with this hypothesized pathway. It appears that two pathways are necessary to induce the antinociceptive effects, and neither is individually sufficient to induce antinociception.

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