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Brain Stem Opioidergic and GABAergic Neurons Mediate the Antinociceptive Effect of Nitrous Oxide in Fischer Rats

Yoko Ohashi, M.D., Tianzhi Guo, M.D.,† Ryo Orii, M.D., Ph.D.,* Mervyn Maze, M.B., Ch.B.,‡ Masahiko Fujinaga, M.D., Ph.D.§*

Background: **Recent studies have revealed that N_2O exerts its antinociceptive effect by inducing opioid peptide release in the brain stem, thereby activating the descending noradrenergic inhibitory neurons, which modulate pain processing in the spinal cord. However, the precise neuronal pathways that mediate these events remain to be determined.**

Methods: **Using immunohistochemical and behavioral techniques in adult male Fischer rats, the authors studied the involvement of brain stem opioidergic and γ-aminobutyric acid–mediated (GABAergic) neurons in the N_2O-induced antinociceptive effect using discrete microinjections of an opioid receptor antagonist or GABAergic activator into the periaqueductal gray area and pontine noradrenergic nuclei. They used c-Fos expression as an immunohistochemical mark of neuronal activation induced by N_2O and the plantar test as the behavioral paradigm for nociception.**

Results: **Microinjection of either naloxone (an opioid receptor antagonist) or muscimol (a γ-aminobutyric acid receptor type A agonist) into the ventrolateral periaqueductal gray area inhibited N_2O-induced c-Fos expression in the spinal cord and pontine noradrenergic nuclei, particularly in the A7. Microinjection of either naloxone or muscimol into the A7 nuclei also inhibited N_2O-induced c-Fos expression in the spinal cord and the N_2O-induced antinociceptive effect by the plantar test.**

Conclusions: **These results support the hypothesis that both opioidergic and GABAergic neurons mediate the antinociceptive effect of N_2O at the periaqueductal gray area and A7 in the brain stem. The authors postulate that N_2O-induced opioid peptide release leads to inhibition of GABAergic neurons *via* opioid receptors. The descending noradrenergic inhibitory pathways, which are tonically inhibited by these γ-aminobutyric acid neurons, are thereby activated (disinhibited) and modulate pain processing in the spinal cord.**

FOR more than a century, N_2O has been used in clinical anesthesia, but the underlying mechanisms of its anesthetic and analgesic properties are not yet fully understood. In 1976, Berkowitz *et al.*[1] reported that the antinociceptive effect of N_2O on the abdominal constriction test in CF-1 mice was inhibited by subcutaneous injection of the opioid receptor antagonists naloxone and naltrexone. It is thought that N_2O induces opioid peptide release in the brain stem, leading to the activation of the descending noradrenergic inhibitory pathways, which results in modulation of the nociceptive processing in the spinal cord.[2] However, the precise neuronal pathways that mediate these events remain to be determined.

The descending inhibitory pathways, which mediate the antinociceptive effect of N_2O, represent an important pain modulating system.[3,4] The periaqueductal gray area (PAG) in the mid-brain of the brain stem integrates the ascending nociceptive input with the descending inhibitory output, which modulates nociceptive processing in the spinal cord. When there is no ascending nociceptive input, the descending inhibitory pathways are tonically inhibited by inhibitory γ-aminobutyric acid–mediated (GABAergic) neurons within the brain stem.[3] Nociceptive input activates the descending inhibitory pathways by removing the tonic inhibition through activation of other inhibitory neurons, such as opioidergic neurons. We posit that N_2O produces the same conditions as those induced by nociceptive input and releases the tonic inhibition on the descending inhibitory pathways.

Based on our earlier studies addressing the N_2O-induced antinociceptive effect, we proposed the neuronal circuitry shown in figure 1.[2] In the current study, we investigated the involvement of brain stem opioidergic and GABAergic neurons in the N_2O-induced antinociceptive effect using discrete microinjections of an opioid receptor antagonist or GABAergic activator into the PAG and pontine noradrenergic nuclei (*i.e.*, A7). We used c-Fos expression as an immunohistochemical marker of neuronal activation induced by N_2O,[5] dopamine β-hydroxylase (DBH; noradrenaline synthesizing enzyme) as an immunohistochemical marker of noradrenergic neurons, and the plantar test as the behavioral paradigm for nociception.

Materials and Methods

Animals

Adult male Fischer rats, in whom N_2O exhibits potent antinociceptive effects,[6] were used (B & K Universal, Grimston Aldbrough Hull, United Kingdom). The study protocol was approved by the United Kingdom Home Office (London), and all animal procedures were carried out in accordance with the United Kingdom (Scientific Procedures) Act of 1986. In addition, all efforts were

* Postdoctoral Fellow, † Research Staff, ‡ Professor, § Senior Lecturer.

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Address reprint requests to Dr. Fujinaga: No. 211, 140 Enshoji-machi, Okazaki, Sakyo-ku, Kyoto 606–8344, Japan. Address electronic mail to: mfujinaga@mac.com. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

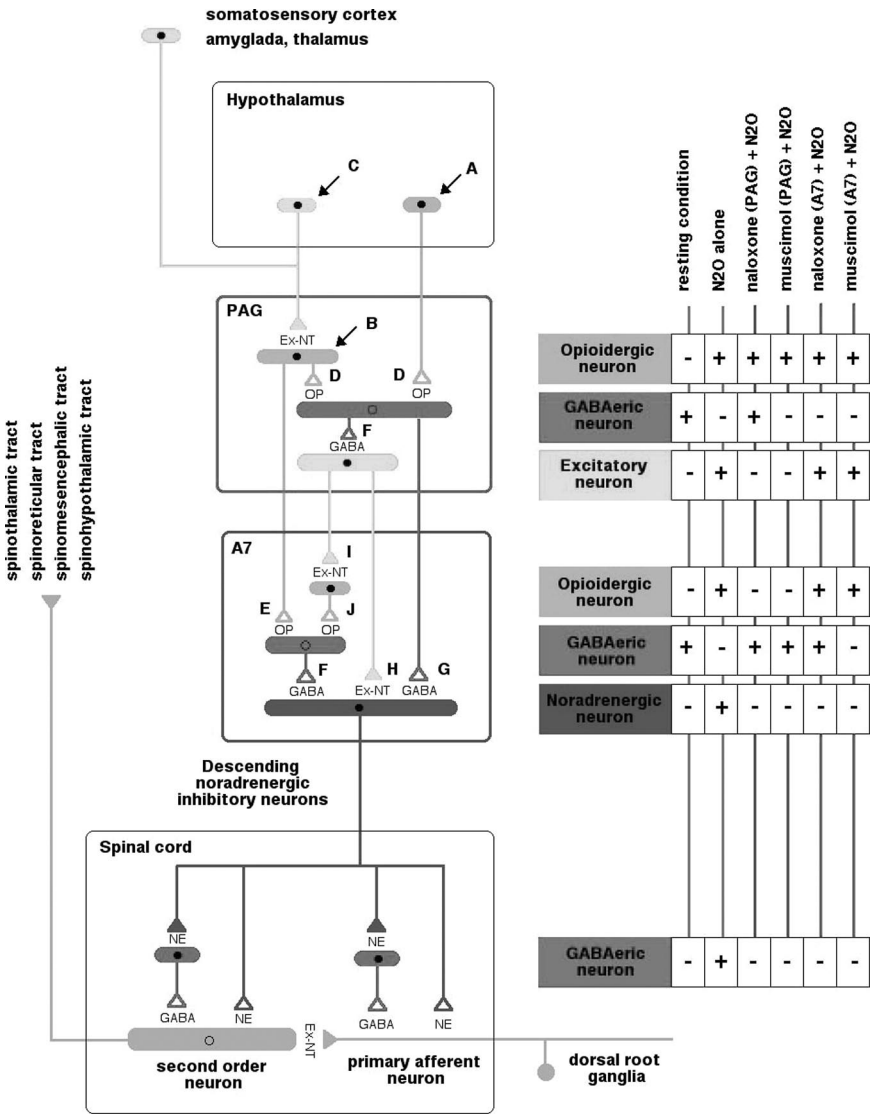

Fig. 1. (*Left*) Putative neuronal pathways involved in the antinociceptive effect of N_2O in rats. *A–J* = putative mechanisms. Ex-NT = excitatory neurotransmitters; GABA = γ-aminobutyric acid; GABAeric = γ-aminobutyric acid–mediated; NE = norepinephrine; OP = opioid peptides; PAG = periaqueductal gray area. *Closed triangles* = excitatory synapses; *open triangles* = inhibitory synapses; *small closed circles* = the nucleus of cells activated by N_2O exposure; *small open circles* = the nucleus of cells inactivated by N_2O exposure. (*Right*) Prediction on neuronal state (+ = activated; − = inactivated) of each cell type at different levels in different groups.

made to minimize animal suffering and reduce the number of animals used.

Chronic Cannulation into the PAG and A7 and Drug Administration

Animals were anesthetized with halothane (3–5%)–air, prepared for aseptic surgery, and secured in a stereotaxis frame. A pair of cannulae (22 gauge, 20 mm length; Tomlinson Tubes, Bidford-on-Avon, United Kingdom) were positioned for bilateral injection into either the ventrolateral PAG (8 mm posterior, 0.6 mm lateral, and 4.8 mm ventral to the surface) or the A7 nuclei (8.8 mm posterior, 2.8 mm lateral, and 7.2 mm ventral to the surface)[7] and fixed with dental resin (Orthoresin; Dentsply, Surrey, United Kingdom). After a minimum of 4 days' recovery, a 30-gauge stainless needle, connected to polyethylene tubing, was inserted into the cannula to enable the needle tip to protrude by 1 mm beyond the tip of the cannula. Saline (0.2 μl × 2), naloxone (0.25 μg/0.2 μl × 2), or muscimol (0.5 μg/0.2 μl × 2) was injected bilaterally at a rate of 0.4 μl/min using a CMA microinjection pump (CMA/100; CMA/Microdialysis, Solna, Sweden). All solutions were injected 15 min before air or N_2O (75%)–O_2 (25%) exposure, which lasted for 90 min. After completing the experiment, the cannula sites were examined to confirm their location.

Gas Exposure

Gas exposure was performed in an acryl plastic chamber (18 inches long, 9 inches wide, and 8 inches high). Air or a mixture of N_2O (75%)–O_2 (25%) was continuously delivered at 4 l/min 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, Herz, United Kingdom). Animals were placed into the chamber through the side door after the desired gas concentrations were achieved and stabilized.

Collection of Spinal Cord and Whole Brain for Immunohistochemical Analysis

After 90 min of gas exposure, which is the time to the peak effect of c-Fos induction by N_2O,[5] the animal was injected intraperitoneally with sodium pentobarbital (100 mg/kg). During this terminal anesthesia, the animal was perfused with 0.1 M phosphate-buffered saline followed by paraformaldehyde, 4%, in 0.1 M phosphate buffer *via* a 16-gauge cannula that was inserted into the ascending aorta through the left ventricle; blood was drained *via* an incision in the right atrium. After perfusion, the animal was decapitated, and the brain was removed and stored in paraformaldehyde, 4%, for 24 h followed by a further 72 h in sucrose, 30%, in 0.1 M phosphate buffer at 4°C. Whole brain was freeze-mounted in embedding matrix and was cut into coronal plane sections (30 μm thick) with a cryostat at −15°C. Sections were collected in phosphate-buffered saline and stored at 4°C.

c-Fos Diaminobenzidine Staining of the Spinal Cord

Free-floating sections of whole brain and spinal cord at the lumber levels (L5) (30 μm thick) were incubated for 1 h at room temperature in blocking solution consisting of rabbit serum, 3%, and Triton X, 0.3%, in phosphate-buffered saline (PBT) and then incubated overnight at 4°C with goat antibody to c-Fos (1:10,000, Cat. No. sc-52-G; Santa Cruz Biotechnology, Santa Cruz, CA) in rabbit normal serum (1%), washed with PBT (three times for 5 min each), and finally incubated for 1 h at room temperature in avidin–biotin–peroxidase complex (VectaStain Elite ABC Kit; Vector Laboratories, Burlingame, CA) in PBT. Visualization of the immunohistochemical reaction was achieved by incubation in diaminobenzidine solution containing nickel ammonium sulfate to which hydrogen peroxide was added (DAB kit; Vector Laboratories). After the staining procedure was completed, the sections were washed in phosphate-buffered saline (three times for 5 min each) followed by 0.1 M phosphate buffer and transferred onto glass slides, which were then dehydrated in ethanol (100%), cleared in xylene (100%), and fitted with a coverslip.

Quantitation of c-Fos-positive Cells in the Spinal Cord

Randomly selected, undamaged sections were photographed using a digital camera (Olympus Digital Camera Model C2020Z; Olympus Optical, Southall, Middlesex, United Kingdom). The number of c-Fos-positive cells was counted for each lamina of the spinal cord (*i.e.*, laminae I to II [superficial area], laminae III to IV [nucleus proprius area], laminae V to VI [neck area], and laminae VII–X [ventral area]), according to the method by Presley *et al.*[8] Five photographs of different sections were taken for each rat, and the mean number of c-Fos-positive cells per section was calculated. Each group had 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 group for counting of c-Fos-positive cells.

Double Immunofluorescent Staining

The brain stem sections containing the noradrenergic cell groups (A7, locus caeruleus, and A5) were collected. Rabbit antibody to DBH (1:2,000, Cat. No. DZ1020; Affiniti Research Products, Mamhead, United Kingdom) immunoglobulins were used for double immunofluorescent staining with c-Fos. Free-floating sections were incubated in blocking solution consisting of normal donkey serum, 3%, in PBT for 1 h at room temperature and in normal donkey serum, 1%, with goat antibody to c-Fos (1:1,000, Cat. No. sc-52-G; Santa Cruz Biotechnology) overnight at 4°C. After incubation, the sections were washed in PBT (three times for 10 min each), incubated in donkey antibody to goat indocarbocyanine immunoglobulin G (1:200; Chemicon International) in normal donkey serum, 1%, for 1 h at room temperature, washed in PBT (three times for 5 min each), and incubated with the above-mentioned primary antibody (rabbit antibody to DBH for two nights) at 4°C. After these procedures, the sections were washed in PBT (three times for 10 min each) and incubated in the secondary antibody (1:200, donkey antibody to sheep or donkey antibody to rabbit fluorescein isothiocyanate–conjugated immunoglobulin G; Jackson ImmunoResearch Laboratories, West Grove, PA). After the staining procedure was completed, the sections were rinsed in phosphate-buffered saline followed by 0.1 M phosphate buffer, transferred onto glass slides, and dried in the darkness. One drop of VectaShield (Vector Laboratories), a mounting medium for fluorescence, was placed on each glass slide, fitted with a coverslip, and examined immediately by fluorescent microscopy (LEICA DMRE microscope; Leica, Wetzlar, Germany).

Analysis of Double Staining Images

Three to four well-preserved, undamaged sections were randomly selected from each animal for analysis. Unlike the A7 and A5 nuclei, in which the DBH-positive cells in each section could be easily counted (< 30 per section), the number of DBH-positive cells in sections of the locus caeruleus was too plentiful to accurately count. In the A7 and A5, colocalization analysis of c-Fos-positive staining in DBH-positive cells was performed using a standard fluorescent microscope. In the locus caeruleus, colocalization of DBH-positive staining in c-Fos-positive cells was performed using a confocal microscopy, which enabled analysis of the section by a 1-μm thickness (Bio-Rad Microradiance MR/AG II System equipped with Argon/Green HeNe lasers; Bio-Rad Laboratories, Hercules, CA). Indocarbocyanine and fluores-

cein isothiocyanate staining were detected separately with E570 LP (red) and HQ500 LP (green) emission filters, respectively. The investigator was blinded to the treatment group during the analysis.

Plantar Test

For a separate set of animals, the plantar test was conducted after 30 min of gas exposure, which coincides with the peak antinociceptive effect of N_2O,[6] using the 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 ≈ 4 s. To avoid tissue damage, a predetermined cutoff time of 10 s was imposed. PWL data consisted of a mean of three trials for each animal. From the PWL, the percentage of the maximal possible effect was calculated as follows: ([PWL with treatment − baseline PWL]/[cutoff time − baseline PWL]) × 100.

Statistical Analysis

The number of c-Fos-positive cells in each lamina was compared using one-way ANOVA followed by *post hoc* Dunn multiple comparison tests. Results from c-Fos and DBH double staining were analyzed using a contingency table, and the Fisher exact test was used as a *post hoc* test. Analysis of the plantar test results was performed using the percentage of the maximal possible effect and by one-way ANOVA and *post hoc* Dunn multiple comparison testing. $P < 0.05$ was considered statistically significant.

Results

Effect of PAG Microinjection on N_2O-induced c-Fos Expression in the Spinal Cord

The total number of c-Fos-positive cells on both sides of the spinal cord section in the air–saline (control) group was ≈ 50–55. Exposure to N_2O significantly increased the number of c-Fos-positive cells in laminae III to IV and V to VI. Microinjection of naloxone or muscimol into the PAG before N_2O exposure significantly decreased the number of N_2O-induced c-Fos-positive cells in laminae III to IV (fig. 2).

Effect of PAG Microinjection on N_2O-induced c-Fos Expression in the A7, A5, and Locus Caeruleus

The incidence of colocalization of c-Fos within DBH-positive cells in the A7 and A5 among the air–saline (control) groups was 30.9% (17 of 55) and 60.3% (25 of 40), respectively; among the saline–N_2O groups, the incidence increased to 90.9% (30 of 33) and 90.3% (28 of 31), respectively (table 1). Representative photomicrographs of colocalization are shown in figure 3. Pretreatment with muscimol into the PAG significantly reduced the incidence of colocalization to 35.0% (14 of 40) and 50.0% (14 of 28) in the A7 and A5, respectively (table 1). Pretreatment with naloxone into the PAG significantly reduced the incidence of colocalization in the A7 (41.2% [14 of 34]) but not in the A5 (77.8% [14 of 18]) (table 1).

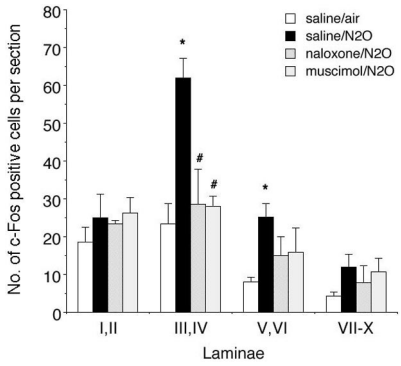

Fig. 2. Effect of PAG-administered naloxone and muscimol on N_2O-induced c-Fos expression in the spinal cord. The number of c-Fos-positive cells in each lamina of the spinal cord at the lumbar level was assessed when saline (0.2 μl × 2), naloxone (0.25 μg/0.2 μl × 2), or muscimol (0.5 μg/0.2 μl × 2) was microinjected into the PAG followed by 90 min of gas exposure with either air or N_2O (75%)–O_2 (25%). *Bar* = mean number for three animals; *error bar* = SD. **P* < 0.05 *versus* saline–air group. #*P* < 0.05 *versus* saline–N_2O group.

The incidence of colocalization of DBH within c-Fos-positive cells in the locus caeruleus among the air–saline (control) groups was 35.4% (11 of 31), and that among the saline–N_2O groups was 97.0% (65 of 67) (table 1). Representative confocal microscopic pictures of double staining are shown in figure 4. Pretreatment with naloxone and muscimol into the PAG significantly reduced the incidence of colocalization to 78.8% (41 of 52) and 78.4% (40 of 51), respectively; these rates were still significantly greater than values seen for the air–saline group (table 1).

Effect of A7 Microinjection on N_2O-induced c-Fos Expression in the Spinal Cord

The total number of c-Fos-positive cells on both sides of the spinal cord section in the air–saline (control) group was ≈ 50–55. Exposure to N_2O significantly increased the number of c-Fos-positive cells in laminae III to IV (fig. 5). Microinjection of naloxone or muscimol into the A7 before N_2O exposure significantly decreased the number of c-Fos-positive cells in laminae III to IV back to that seen in the control group (fig. 5).

Effect of A7 Microinjection on the N_2O-induced Antinociceptive Effect on the Plantar Test

The baseline PWL was ≈ 4.0–4.4 s in each group and was unaltered by saline administration (fig. 6). Rats exposed to N_2O showed a significant increase in PWL (fig.

Table 1. Number of c-Fos-positive Cells among DBH-positive Cells in the A7 and A5 and Number of DBH-positive Cells among c-Fos-positive Cells in the Locus Caeruleus

Pretreatment			Animal Number				
Drug	Dose	Exposure	1	2	3	4	Total
A7							
Saline	0.2 μl × 2	Air	0/3	7/25	10/27	—	17/55 (30.9%)
Saline	0.2 μl × 2	N_2O,75%	3/3	10/10	11/12	6/8	30/33 (90.9%)*
Naloxone	0.25 μg/0.2 μl × 2	N_2O,75%	1/3	6/18	5/7	2/6	14/34 (41.2%)†
Muscimol	0.5 μg/0.2 μl × 2	N_2O,75%	11/15	3/20	0/5	—	14/40 (35.0%)†
A5							
Saline	0.2 μl × 2	Air	3/8	10/16	12/16	—	25/40 (60.3%)
Saline	0.2 μl × 2	N_2O,75%	6/8	11/12	11/11	—	28/31 (90.3%)*
Naloxone	0.25 μg/0.2 μl × 2	N_2O,75%	3/5	3/8	5/5	—	14/18 (77.8%)
Muscimol	0.5 μg/0.2 μl × 2	N_2O,75%	6/13	7/8	1/7	—	14/28 (50.0%)†
Locus caeruleus							
Saline	0.2 μl × 2	Air	1/12	6/12	4/7	—	11/31 (35.4%)
Saline	0.2 μl × 2	N_2O,75%	13/13	20/22	32/32	—	65/67 (97.0%)*
Naloxone	0.25 μg/0.2 μl × 2	N_2O,75%	27/29	8/8	6/15	—	42/52 (78.8%)*†
Muscimol	0.5 μg/0.2 μl × 2	N_2O,75%	19/21	15/20	6/10	—	40/51 (78.4%)*†

* $P < 0.05$ vs. saline–air; † $P < 0.05$ vs. saline–N_2O.

DBH = dopamine β-hydroxylase.

6). Pretreatment with either naloxone or muscimol into the A7 significantly attenuated the increased latency seen with N_2O (fig. 6).

Discussion

The PAG and noradrenergic nuclei in the brain stem integrate the ascending nociceptive input with the descending inhibitory output.[3,4,9,10] The involvement of the PAG in the N_2O-induced antinociceptive effect has been suggested by several previous studies. In 1987, Zuniga *et al.*[11] reported that the kainic acid–induced lesion of the PAG almost completely attenuated the antinociceptive effects of N_2O by the tail and foot flick tests in Sprague-Dawley rats. In 1994, Hodges *et al.*[12] reported that unilateral microinjection of D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, a μ-opioid receptor antagonist, into the PAG partially blocked the antinociceptive effects of N_2O in a dose-dependent manner by the hot plate test in Sprague-Dawley rats. More recently, Fang *et al.*[13] reported in 1997 that bilateral microinjection of naloxone (2.5 μg/0.5 μl saline), but not yohimbine (1.5 μg/0.5 μl saline), an α_2-adrenoceptor antagonist, into the ventrolateral PAG partially blocked the antinociceptive effects of N_2O by the tail flick test in Sprague-Dawley rats. Collectively, data from these studies support the tenet that the PAG and, more specifically, opioid receptors within the PAG are involved in mediating the antinociceptive effects of N_2O.

The involvement of the descending noradrenergic inhibitory neurons in the N_2O-induced antinociceptive effect had been circumstantially alluded to in the previous

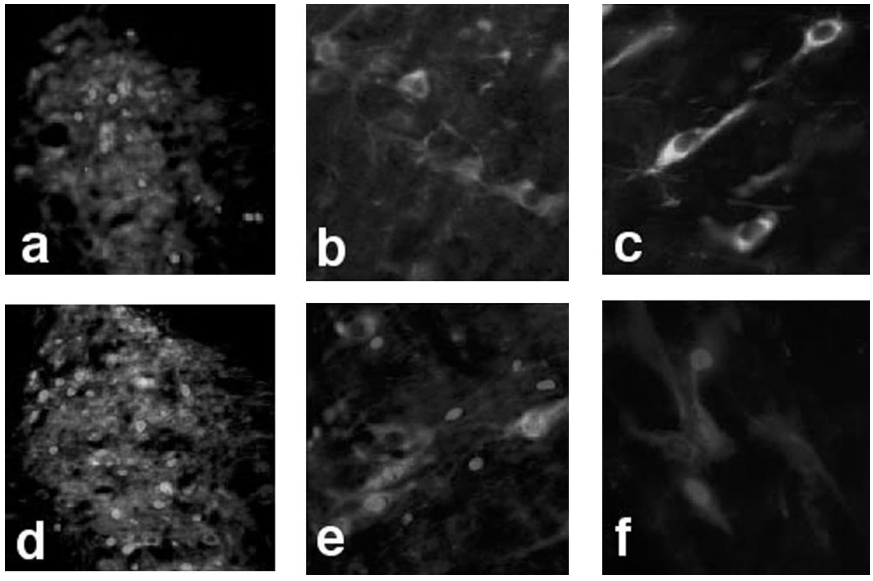

Fig. 3. Representative photomicrographs of double fluorescent staining. Sections of the pontine noradrenergic nuclei, *i.e.*, locus caeruleus (*a, d* [low magnification]), A7 (*b, e* [high magnification]), and A5 (*c, f* [high magnification]), were stained for both c-Fos and dopamine β-hydroxylase after exposure to air (*a–c*) or N_2O (*d–f*).

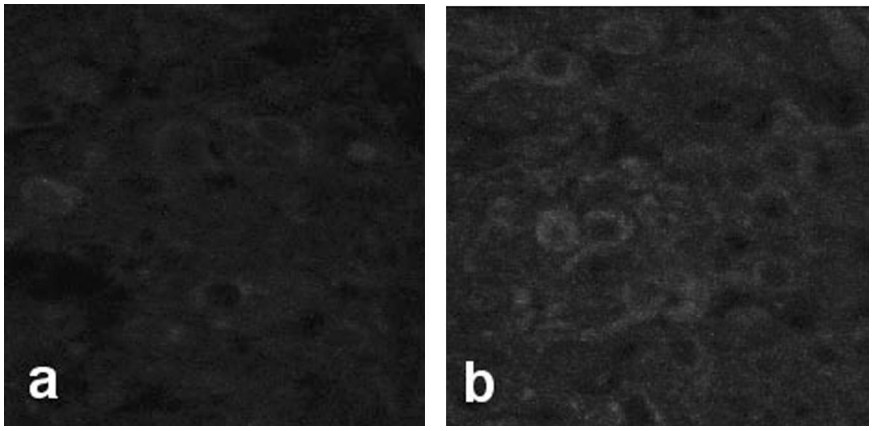

Fig. 4. Representative confocal microscopic pictures of sections of the locus caeruleus double stained for c-Fos and dopamine β-hydroxylase in saline–air (*a*) and saline–N_2O (*b*) groups.

studies. Both systemically[14] and intrathecally[15] administered α_2-adrenoceptor antagonists blocked the antinociceptive effects of N_2O by the tail flick test in Sprague-Dawley rats. In 1999, Zhang *et al.*[16] reported that N_2O increased the norepinephrine release into the dorsal horn of the spinal cord in Sprague-Dawley rats. Furthermore, if norepinephrine in the spinal cord is depleted by intrathecal injection of *n*-(2-chloroethyl)-*n*-ethyl-2-bromobenzylamine, N_2O no longer showed the antinociceptive effects by the tail flick test.[16] In 1998, Fukuhara *et al.*[17] reported that the lesion of locus caeruleus created by electrical coagulation attenuated the antinociceptive effect of N_2O by the tail flick test in Wistar rats. In 2000, Sawamura *et al.* [18] reported that N_2O activated the pontine noradrenergic nuclei in Sprague-Dawley rats (A7, locus caeruleus, and A5) using c-Fos as an immunohistochemical marker of neuronal activation. When the rats were microinjected intracerebroventricularly with the mitochondrial toxin saporin coupled to antibody to DBH, ≈ 70% of the noradrenergic cells in these nuclei were destroyed, and the animals no longer showed N_2O-induced antinociceptive effects by the tail flick test. Most recently, we showed that N_2O administration induced c-Fos expression in the spinal cord, especially in laminae III to IV, as a result of activation of the descending noradrenergic inhibitory neurons.[5] Subsequently, we reported that N_2O-induced c-Fos expression was localized to GABAergic neurons in the spinal cord, which were activated by α_1 adrenoceptors.[19]

Based on these studies and the neuronal pathways in the brain stem, we proposed neuronal circuits that may be involved in the activation of the descending noradrenergic pathways by N_2O (fig. 1). We posit that N_2O-induced opioid peptide release disinhibits the descending noradrenergic inhibitory neurons by one or more of the following mechanisms: direct activation of opioidergic neurons that innervate the PAG (fig. 1, *A*), direct activation of opioidergic neurons within the PAG (fig. 1, *B*), and indirect activation of opioidergic neurons within the PAG through excitatory neurons from other sites (fig. 1, *C*). The PAG is rich in local opioidergic interneu-

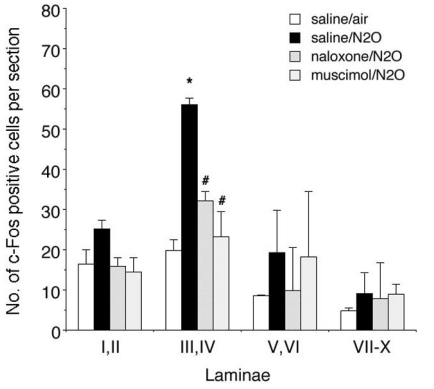

Fig. 5. Effect of A7 administered naloxone and muscimol on the number of N_2O-induced c-Fos-positive cells in each lamina of the spinal cord. Rats were microinjected into the A7 bilaterally with saline (0.2 μl × 2), naloxone (0.25 μg/0.2 μl × 2), or muscimol (0.5 μg/0.2 μl × 2). This was followed by 90 min of gas exposure to either air or N_2O (75%)–O_2 (25%), and the c-Fos-positive cells were counted in the laminae of the spinal cord at the lumbar level. *Bar* = mean number for three animals; *error bar* = SD. **P* < 0.05 *versus* saline–air group. #*P* < 0.05 *versus* saline–N_2O group.

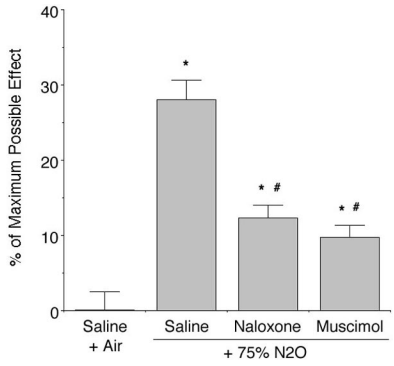

Fig. 6. Effect of A7 administered naloxone and muscimol on N_2O-induced antinociception. The plantar test for withdrawal latency was assessed when saline (0.2 μl × 2), naloxone (0.25 μg/0.2 μl × 2), or muscimol (0.5 μg/0.2 μl × 2) was microinjected into the A7, which was followed by 30 min of exposure to either air or N_2O (75%)–O_2 (25%). The latency is expressed as the percentage of the maximum possible effect. *Bar* = the mean number for four animals; *error bar* = the SD. **P* < 0.05 *versus* saline–air group. #*P* < 0.05 *versus* saline–N_2O group.

rons, thus the opioid peptides released in the PAG are most likely to inhibit the GABAergic neurons within the PAG (fig. 1, *D*). Nevertheless, it is also possible that N_2O activates the opioidergic neurons in the PAG that result in inhibition of the GABAergic inhibitory interneurons in the A7 through opioid receptors (fig. 1, *E*), because some enkephalinergic neurons in the ventrolateral PAG have been shown to directly project to the A7, where the target cells have been identified as nonnoradrenergic neurons.[20,21]

Consequently, the manner whereby GABAergic inhibitory interneurons disinhibit the descending noradrenergic pathways in the A7 may involve the following: opioidergic neurons projecting from the PAG to GABAergic inhibitory interneurons in the A7 (fig. 1, *F*), GABAergic inhibitory interneurons projecting from the PAG directly to the noradrenergic neurons in the A7 (fig. 1, *G*), and GABAergic inhibitory interneurons innervating excitatory interneurons within the PAG that project onto the noradrenergic neurons in the A7 (fig. 1, *H*).[22] In addition, because local opioidergic interneurons have been identified within the A7, there is another possible pathway: the excitatory neurons in the PAG projecting to the opioidergic inhibitory interneurons in the A7 (fig. 1, *I*), which innervate the GABAergic inhibitory interneurons in the A7 (fig. 1, *J*), thereby inhibiting the noradrenergic neurons (fig. 1, *F*).

In the current study, we first examined the effect of naloxone, an opioid receptor antagonist, and muscimol, a γ-aminobutyric acid receptor type A agonist, microinjected into the PAG. Both naloxone and muscimol attenuated N_2O-induced c-Fos expression in laminae III to IV (fig. 2), indicating that both agents inhibited the activity of the descending noradrenergic inhibitory neurons within the PAG. Because tonic inhibition of the descending noradrenergic inhibitory neurons was reestablished by this pretreatment, we suggest that the sites of action are at *D* and *F* on figure 1. Although the results from naloxone experiments corroborate findings of the previous studies,[12,13] those from muscimol experiments are novel and provide a new insight on the involvement of GABAergic neurons in the PAG in the N_2O-induced antinociceptive effect.

As a next step, we examined the effect of naloxone and muscimol microinjected into the PAG on N_2O-induced c-Fos expression in three major noradrenergic nuclei in the brain stem, *i.e.*, A7, locus caeruleus, and A5 (table 1). Pretreatment with both agents in the PAG restored c-Fos expression in the A7 to control level. However, the effects of pretreatment in the PAG less completely attenuated N_2O-induced c-Fos expression in both the locus caeruleus and the A5. For example, in the locus caeruleus, both agents only partially attenuated N_2O-induced c-Fos expression. In the A5, muscimol significantly attenuated N_2O-induced c-Fos expression almost to the baseline level, but naloxone showed almost no effect. Because no dose–response studies were conducted, we are unable to distinguish whether the A7 differs from the A5 and locus caeruleus, qualitatively or quantitatively.

If these represent qualitative differences, we suggest that the A7 nucleus is the most likely to be involved in the antinociceptive effect of N_2O. Noradrenergic neurons in the A7 project to laminae I–IV in the spinal cord, which are the sites of nociceptive processing.[23] Descending noradrenergic projections from either the locus caeruleus or the A5 appear not to terminate in regions of importance for nociceptive processing. For example, the locus caeruleus neurons mostly project to laminae VII to VIII (ventral horn) and IX to X (motor neurons),[24] although strain differences of such neuronal projections have been reported.[25–28] Those from the A5 project to laminae IV to VII and X and the intermediolateral cell column.[29] These laminae are not directly involved in nociceptive processing. Rather, noradrenergic projections from the locus caeruleus may be involved in the hypnotic effects of N_2O,[30–32] whereas those from the A5 are involved in the cardiovascular effects of N_2O.[33,34]

Because of the results from the PAG pretreatments, we focused further investigations on the A7 to assess its role in the activation of the spinal cord neurons on the antinociceptive effect of N_2O. Pretreatment with both naloxone and muscimol in the A7 attenuated N_2O-induced c-Fos expression most significantly in laminae III to IV (fig. 5). Furthermore, both pretreatments attenuated the antinociceptive effect of N_2O assessed by the plantar test (fig. 6). These findings establish the involvement of the A7 in the N_2O-induced antinociceptive effect and support the notion that opioidergic and GABAergic neurons are mediating this effect in the A7 at sites *E* or *J* and *F* or *G* on figure 1, respectively.

Taken together, we suggest that N_2O-induced opioid peptide release leads to inhibition of GABAergic neurons *via* opioid receptors by multiple mechanisms that work in concert. The descending noradrenergic inhibitory neurons, which are tonically inhibited by γ-aminobutyric acid neurons, are activated and modulate pain processing in the spinal cord. Several possible mechanisms mediate the antinociceptive effect of N_2O, as shown in figure 1. The relative importance of each neuronal circuit on the antinociceptive effect of N_2O remains to be clarified, as does the initial site of action by N_2O and the mechanism underlying opioid peptide release by N_2O.

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