Anesthesiology 2003; 99:708–15

Corticotropin-releasing Factor Mediates the Antinociceptive Action of Nitrous Oxide in Rats

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Background: **Exposure to nitrous oxide activates brainstem noradrenergic nuclei and descending inhibitory pathways, which produce the acute antinociceptive action of nitrous oxide. Because corticotropin-releasing factor (CRF) can produce activation of noradrenergic neurons in the locus ceruleus, the authors sought to determine whether it might be responsible for the antinociceptive action of nitrous oxide.**

Methods: **Male Sprague-Dawley rats (250–300 g) were exposed for 60 min to room air or 25, 50 or 70% nitrous oxide in oxygen. Brain sections including the hypothalamus were immunostained for both c-Fos (a marker of neuronal activation) and CRF and the percentage of CRF-positive neurons expressing c-Fos was determined. The functional consequences of changes in CRF were investigated by assessing the effect of intracerebroventricular administration of a CRF antagonist (α-helical CRF_{9-41}, 20 μg/10 μl) on both activation of locus ceruleus noradrenergic neurons and the antinociception (with the tail-flick latency test) produced by nitrous oxide.**

Results: **Inhalation of nitrous oxide induced a dose-dependent increase in c-Fos expression in CRF-positive neurons in the paraventricular nucleus of the hypothalamus. Intracerebroventricular administration of CRF antagonist inhibited nitrous oxide–induced c-Fos expression in the locus ceruleus and the antinociceptive effect of nitrous oxide.**

Conclusions: **Nitrous oxide activates the CRF system in the brain, which results in stimulation of noradrenergic neurons in the locus ceruleus and its consequent antinociceptive effect.**

ALTHOUGH the mechanism underlying the analgesic action of nitrous oxide is still unclear, involvement of descending noradrenergic inhibition has been strongly suggested. Nitrous oxide activates noradrenergic neurons in the rat brainstem[1] and increases noradrenaline release in the brain and the spinal cord.[2,3] Both antagonism of adrenergic receptors[4] and depletion of noradrenaline[3] in the spinal cord inhibit the antinociceptive effect of nitrous oxide. Furthermore, lesioning of brainstem noradrenergic neurons also inhibits nitrous oxide antinociception.[1] These findings suggest that nitrous oxide exerts its antinociceptive effect, at least in part, *via* the activation of descending noradrenergic inhibitory pathways. However, the mechanisms of activation of noradrenergic neurons by nitrous oxide remain unclear.

Corticotrophin-releasing factor (CRF) is released in response to various types of stressors and is a key mediator of the behavioral, endocrinologic, and physiologic responses to stressors.[5,6] In addition to its well-described effect on the pituitary gland, CRF acts as a neurotransmitter and activates diverse intracellular signaling pathways.[7] The CRF system is reported to stimulate the locus ceruleus (LC) neurons through direct innervation[8,9] resulting in the activation of the noradrenergic neuron system in the brain.[10] Furthermore, CRF exerts an antinociceptive action by central and peripheral mechanisms, possibly *via* activation of descending noradrenergic systems.[6,11] In this study, we tested the hypothesis that the CRF system in the brain may be involved in the activation by nitrous oxide of the LC neurons and descending noradrenergic-inhibitory pathways.

Using immunohistochemical expression of c-Fos protein as a marker of neuronal activation,[12] we sought to determine whether nitrous oxide activated CRF-containing neurons in the paraventricular nucleus (PVN) of the hypothalamus, a prominent site of CRF production. We then examined the effect of intracerebroventricular administration of a CRF antagonist, α-helical CRF_{9-41}, on nitrous oxide–induced activation of noradrenergic neurons in the LC (as reflected by c-Fos expression). Lastly, to determine whether CRF mediated the antinociceptive action of nitrous oxide, we studied the effect of the CRF antagonist on the prolongation of the tail-flick latency by nitrous oxide.

Materials and Methods

Animals

These experiments were reviewed and approved by Tokyo University Ethical Committee on Animal Research (Tokyo, Japan). Male Sprague-Dawley rats (250–300 g) were housed in a temperature-controlled and humidity-controlled environment and were maintained on a 12-h light/dark cycle. Food and water were available *ad libitum*. Rats had been habituated to the experimental condition for 2 h for each of 5 consecutive days. All of the behavioral experiments, tissue sampling, and habituation were performed between 8 and 11 AM

Reagents

The CRF antagonist, α-helical CRF_{9-41}, was purchased from Sigma-Aldrich Japan (Tokyo, Japan) and dissolved in 1% acetic acid with a final concentration of 2 μg/μl. We selected a dose of 20 μg of α-helical CRF_{9-41} as has been previously reported.[13,14] Dexmedetomidine was kindly provided by Abbott Japan (Tokyo, Japan).

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Received from the Department of Anesthesiology, Tokyo University Hospital, Tokyo, Japan. Submitted for publication March 7, 2003. Accepted for publication May 28, 2003. Support was provided solely from institutional and/or departmental sources.

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Immunohistochemistry

Rats were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) and transcardially perfused with 100 ml of 0.1 M sodium phosphate-buffered saline, followed by 500 ml of 2% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer cooled to 4°C. After decapitation, the whole brain was removed and immersed in the same fixative for 24 h at 4°C. Tissues were then stored overnight in 30% sucrose solution in 0.1 M phosphate buffer at 4°C for cryoprotection. The brain was sliced into 40-μm-thick sections with a cryotome (CM1800, Leica, Heidelberg, Germany) at −15°C. Every third section including the PVN or the LC was retained and placed in phosphate-buffer solution.

Sections were then double-stained for c-Fos and either CRF or tyrosine hydroxylase (TH). Sections were first incubated for 1 h in blocking solution (5% normal rabbit serum and 0.3% Triton X in phosphate-buffered saline) and then incubated overnight with goat anti–c-Fos antibody (1:5,000, Santa-Cruz Biotechnology, Santa-Cruz, CA) diluted in 1% normal rabbit serum and 0.3% Triton X in phosphate-buffered saline (buffer 1). After vigorous rinsing in buffer 1, sections were incubated for 1 h in biotinylated rabbit anti–goat immunoglobulin (1:200, Chemicon, Temecula, CA) in buffer 1. Sections were vigorously rinsed with 0.3% Triton X in 0.1 M phosphate-buffered saline (buffer 2) and then incubated for 1 h in avidin-biotin-peroxidase complex (Vectra Elite ABC, Vector Laboratories, Burlingame, CA) in buffer 2. Visualization of the reaction product was achieved by incubation for 4 min with diaminobenzidine and nickel-ammonium sulfate in the presence of hydrogen peroxide (diaminobenzidine kit, Vector Laboratories). Sections were rinsed in phosphate buffer and incubated in either goat anti-CRF antibody (1:1,000, Santa Cruz Biotechnology) or anti-TH antibody (1:3,000, Santa Cruz Biotechnology) using the same procedures as for c-Fos immunostaining except that the diaminobenzidine reaction was performed for 2 min in the absence of nickel-ammonium sulfate. As a result, c-Fos positive nuclei were stained black with cytoplasmic CRF or TH expression appearing brown. All incubations were performed at room temperature. After these staining procedures, the sections were rinsed in water and placed on a glass slide. The sections were dehydrated, cleared in 100% xylene, and covered. Care was taken to process samples from different groups simultaneously to minimize the effect of fluctuation in staining.

Tail-flick Latency Test

Tail-flick latencies (TFL) were determined from the mean of three consecutive latencies using a tail-flick apparatus (Muromachi Ikakikai, Tokyo, Japan). The interstimulus interval was approximately 1 min. A high-intensity light was focused on the ventral surface of the middle third of the tail, and the time for the animal to move its tail out of the light beam was automatically recorded. Three different sites of the middle third of the tail were exposed to the light beam to minimize the risk of tissue damage. The same light stimulus intensity was used in all experiments and was preset to give a mean latency of 2.5 s under room air. A cutoff time of 6 s was used to avoid the possibility of tissue damage. If no response had occurred by this time, a value of 6 s was ascribed to the test.

Tail-skin temperature was measured immediately before the TFL test with a thermocouple probe mounted on a heat insulating plate (2 × 2 cm). For temperature measurement, the probe was kept in contact with the ventral surface of the tail close to the point heated in the TFL test. The rats were kept on a heating blanket throughout the TFL tests to maintain the tail temperature within 1° of 30°C.

Intracerebroventricular Cannulation

Rats were anesthetized with intraperitoneal pentobarbital (65 mg/kg) and placed in a stereotaxic apparatus (Muromachi Ikakikai). The skin of the skull was incised in the midline and a hole was drilled (1.5 mm lateral and 1.0 mm posterior to the bregma). A 27-gauge guide cannula (4.0 mm length; Plastics One, Roanoke, VA), inserted with a stylet, was implanted into the left lateral ventricle and fixed to the skull with dental cement and two screws. After the surgical procedure, rats were housed individually for 7 days before experiments were performed. The correct placement of the intracerebroventricular catheter was verified after the experiments by histologic examination of the fixed brain. Only data from animals with correct placement of the cannulae were included.

Intracerebroventricular Administration of CRF Antagonist

The internal cannula was connected to PE-20 tubing (25 cm) with a three-way stopcock attached on the other end. The tubing and the internal cannula were initially filled with distilled water and then end-loaded either with α-helical CRF_{9-41} (20 μg/10 μl) or with vehicle using a microsyringe attached to the three-way cock. A small air bubble was placed between the distilled water and the substance to be injected. Rats were anesthetized briefly with halothane and the internal cannula was inserted down through the guide cannula. The three-way cock was opened to the atmosphere and lifted up so that the solution in the PE tubing was delivered into the ventricle by hydrostatic pressure. Either α-helical CRF_{9-41} or vehicle was injected over a 1-min period and the internal cannula was withdrawn 1 min later. If the solution did not descend by hydrostatic pressure, it was assumed not to be intraventricular and data from the rat were not further analyzed.

Nitrous Oxide and c-Fos Expression in the CRF-positive Neurons of the PVN

Rats were individually exposed for 60 min to room air or 25, 50, or 70% nitrous oxide with oxygen in a Plexiglas chamber (25 × 25 × 30 cm, n = 7 per group). An airway gas monitor (Model 254, Datex, Helsinki, Finland) continuously monitored the concentrations of oxygen, nitrous oxide, and carbon dioxide in the chamber, and flow rates were adjusted to maintain the desired concentrations. After 60 min of gas exposure, rats were anesthetized, perfused with fixative, and the brains were removed as described previously. Every third brain section including the PVN (coordinates: 1.5–2.0 mm caudal to the bregma, 0.5–1.0 mm lateral to the midline and 5.5–6.5 mm below the dura) was picked up and double immunostained for c-Fos and CRF expression.

CRF Antagonist and Nitrous Oxide–induced c-Fos in the LC

One week after intracerebroventricular cannulation, rats underwent pretreatment with intracerebroventricular administration of vehicle (1% acetic acid) or α-helical CRF_{9-41} (20 μg/10 μl) during brief halothane anesthesia. Twenty minutes later, rats were exposed to 70% nitrous oxide and 30% oxygen or air for 60 min in a Plexiglas chamber (n = 7 for each of the four groups). The animals were then perfused and fixed as described previously. Every third section of the midbrain was picked up and double immunostained for c-Fos and TH expression.

CRF Antagonist and the Antinociceptive Action of Nitrous Oxide

In a different group of rats (n = 7), baseline measurement of TFL ($TFL_{baseline}$) was performed 20 min after intracerebroventricular injection of α-helical CRF_{9-41} or vehicle (1% acetic acid) after complete recovery from brief halothane anesthesia. The animals were then transferred into a chamber filled with 70% nitrous oxide and 30% oxygen. After 20 min of gas exposure, an examiner blinded to the intracerebroventricular pretreatment performed the TFL measurement (TFL_{N2O}). On separate occasions with a 1-week interval, individual rats were tested after each pretreatment (α-helical CRF_{9-41} and the vehicle) performed in a random order. Next, to determine whether CRF system is selectively involved in nitrous oxide analgesia, the effect of α-helical CRF_{9-41} on the antinociception by dexmedetomidine, a novel α2-adrenergic agonist, was tested in a different group of rats (n = 7). Administration of α2-adrenergic agonist is associated with *decrease* in the expression of c-Fos in the LC[15]; hence, the CRF antagonist would not be expected to block the antinociceptive effect. Baseline measurement of TFL ($TFL_{baseline}$) was performed 20 min after intracerebroventricular injection of α-helical CRF_{9-41} or vehicle. TFL measurement was repeated 20 min after intraperitoneal administration of 50-μg/kg dexmedetomidine (TFL_{DEX}). On separate occasions with a 1-week interval, individual rats were tested after each pretreatment (α-helical CRF_{9-41} and the vehicle) performed in a random order.

Statistical Analysis

Three sections containing the largest number of CRF-positive neurons in the PVN were selected in each rat. An examiner who was blinded to the treatment given to the rat scored and averaged the percentage of CRF-positive neurons in the PVN that exhibited c-Fos expression. Data were analyzed using the ANOVA and Bonferroni tests. Of the 10 sections containing the TH-positive LC neurons, the three with the largest number of c-Fos–positive nuclei were counted and aggregated by an investigator blinded to the treatment. Data were analyzed with the ANOVA and Bonferroni tests. TFL data are expressed as percent maximal possible effect (MPE) obtained from the following equation: $(TFL_{N2O\ or\ DEX} - TFL_{baseline})/(6 - TFL_{baseline}) \times 100$, where *6* is the cutoff latency. Percent MPE and $TFL_{baseline}$ values after each pretreatment protocol were compared using the paired *t* test. All the data were expressed as mean ± SD and *P* values less than 0.05 were considered significant.

Results

Nitrous Oxide Induces c-Fos in the CRF-positive Neurons of the PVN

In the PVN of the hypothalamus, CRF-positive cytoplasm was stained brown and c-Fos–positive cell nuclei were stained black after immunohistochemical processing (fig. 1). Expression of c-Fos was very limited or absent in rats exposed to room air (fig. 1B). Exposure to 70% nitrous oxide induced a significant increase of c-Fos expression in CRF-positive neurons of the PVN (fig. 1C). In rats exposed to 25, 50, and 70% nitrous oxide, the percentage of CRF-positive neurons in the PVN that expressed c-Fos was 16 ± 3, 32 ± 4, and 86 ± 17%, respectively (fig. 2). In rats exposed to 70% nitrous oxide, c-Fos–positive neurons were also identified in other regions of the brain including the thalamic and cortical nuclei, LC, parabrachial nucleus, periaqueductal gray, and cuneiform nucleus. c-Fos expression was not induced by nitrous oxide in CRF-positive neurons of the stria terminalis and central nucleus of the amygdala, two other prominent regions of CRF production.

Intracerebroventricular Administration of CRF Antagonist Attenuates Nitrous Oxide–induced c-Fos in the LC

In the pontine brainstem sections, TH-positive cytoplasm in the LC was stained brown and c-Fos–positive cell nuclei were stained black after immunohistochemi-

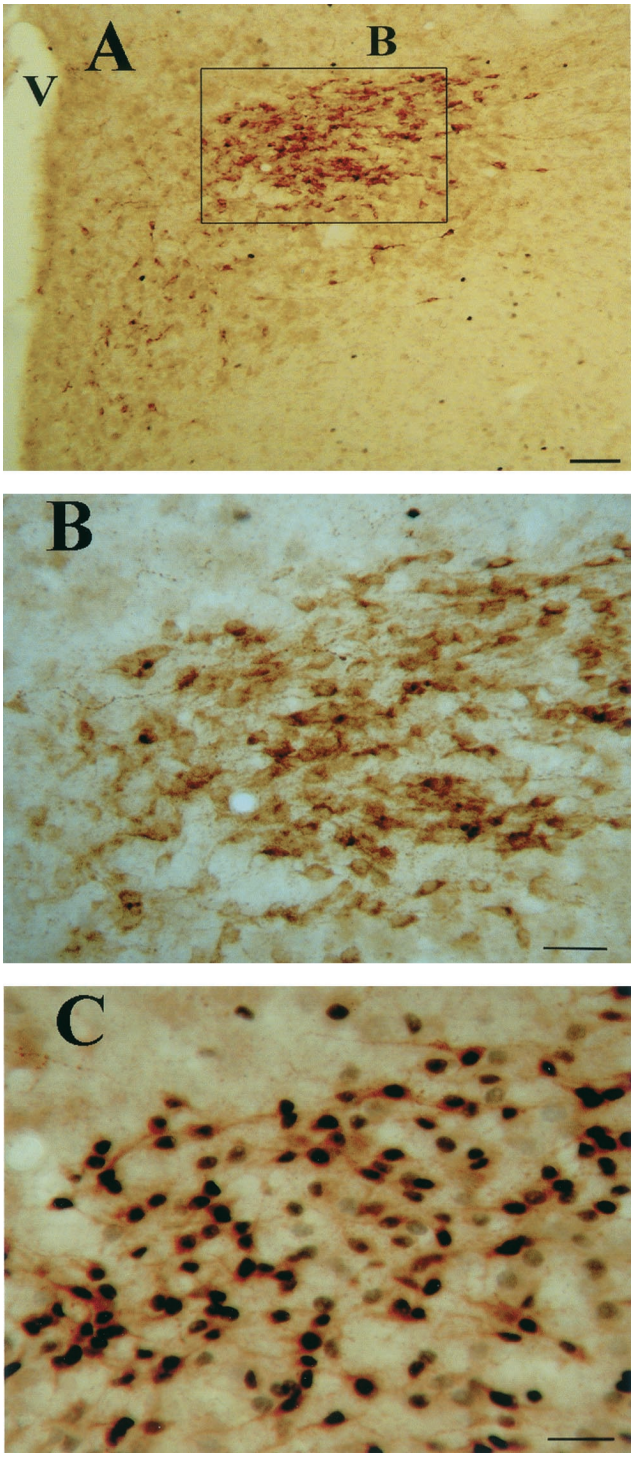

Fig. 1. c-Fos induction in corticotropin-releasing factor (CRF)-containing neurons of the paraventricular nucleus of the hypothalamus. (*A*) The third ventricle and the CRF-positive neurons in the paraventricular nucleus. CRF-containing cytoplasm and c-Fos–positive nuclei are immunohistochemically stained brown and black, respectively. (*B*) Magnification of *panel* A. After 60 min of exposure to air, very little or no c-Fos–positive nuclei were evident in CRF-containing neurons. (*C*) After 60-min exposure to 70% nitrous oxide, c-Fos–positive nuclei are identifiable in most of the CRF-containing neurons. V = third ventricle. *Scale bars* = 100 μm (*A*), 20 μm (*B* and *C*).

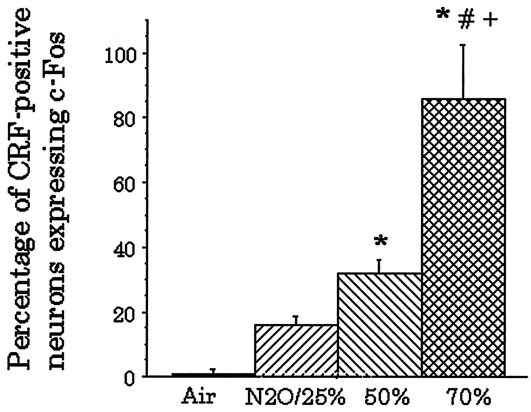

Fig. 2. Percentage of corticotropin-releasing factor (CRF)-positive paraventricular nucleus neurons expressing c-Fos in rats exposed for 60 min to room air or 25, 50, or 70% nitrous oxide (mean ± SD). **P* < 0.0005 *versus* air, #*P* < 0.0001 *versus* N_2O/25%, +*P* < 0.0001 *versus* N_2O/50%.

cal processing (fig. 3). Expression of c-Fos in the LC was not identifiable after air exposure in the vehicle or CRF-antagonist group (fig. 3A and B). Marked c-Fos expression was induced in the LC by exposure to 70% nitrous oxide in rats pretreated with intracerebroventricular vehicle (figs. 3C and 4). Nitrous oxide–induced c-Fos expression was significantly inhibited by intracerebroventricular pretreatment with α-helical CRF_{9-41} (figs. 3D and 4).

Intracerebroventricular Administration of CRF Antagonist Attenuates the Antinociceptive Action of Nitrous Oxide

There was no significant difference in baseline TFL values between the two pretreatments (2.6 ± 0.3 *vs.* 2.4 ± 0.2 s). The antinociceptive effect of nitrous oxide, evaluated by the percent MPE in the TFL test, was significantly inhibited by intracerebroventricular pretreatment with α-helical CRF_{9-41} compared with the vehicle (2 ± 10 *vs.* 40 ± 26%, $P < 0.05$). These data indicate that prolongation of TFL latency by exposure to nitrous oxide was hardly detectable in rats pretreated with α-helical CRF_{9-41}. However, the antinociceptive effect of intraperitoneal dexmedetomidine was not inhibited by intracerebroventricular pretreatment with α-helical CRF_{9-41} as compared with the vehicle (MPE: 85 ± 12 *vs.* 81 ± 10%).

Discussion

In this study, we have demonstrated that nitrous oxide activates CRF-positive neurons in the PVN as evidenced by c-Fos expression. Intracerebroventricular administration of a CRF antagonist, α-helical CRF_{9-41}, inhibited c-Fos expression, and hence the activation of the LC neurons by nitrous oxide exposure. The CRF antagonist also inhibited the antinociceptive effect of nitrous oxide

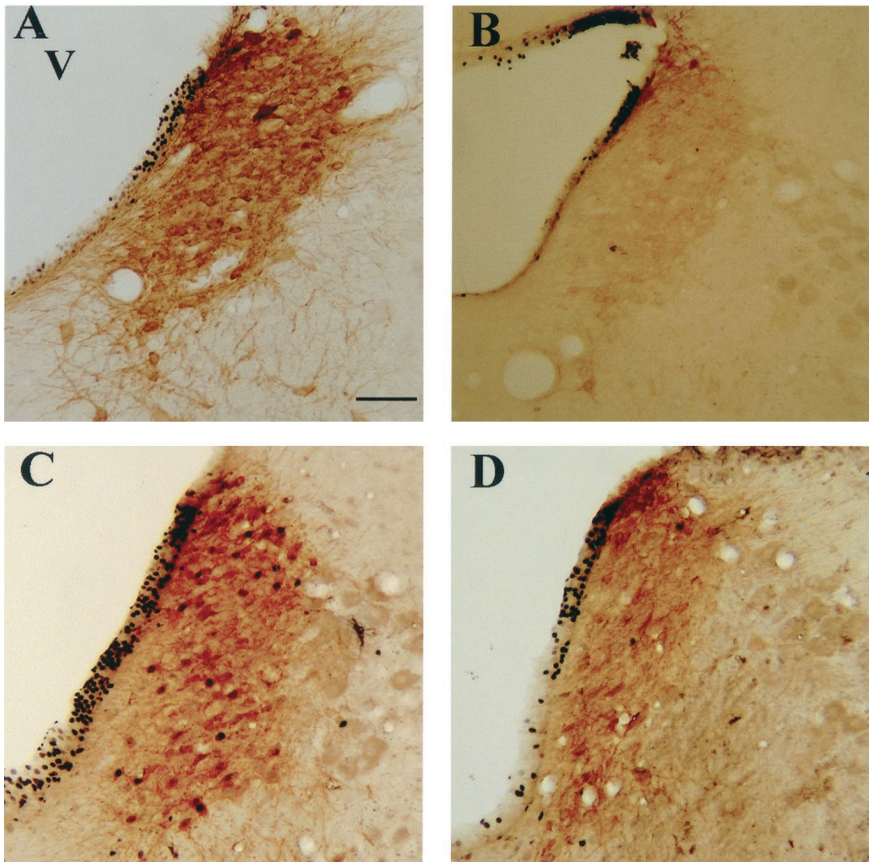

Fig. 3. c-Fos induction in the locus ceruleus after 60-min exposure to air or 70% nitrous oxide. Noradrenergic neurons in the locus ceruleus are immunostained brown using an antibody directed against tyrosine hydroxylase. Air exposure induced very few c-Fos in the locus ceruleus in rats pretreated with intracerebroventricular vehicle (*A*) or α-helical CRF_{9-41} (*B*). (*C*) Nitrous oxide induced remarkable c-Fos expression in the locus ceruleus in rats pretreated with intracerebroventricular vehicle. (*D*) Intracerebroventricular pretreatment with α-helical CRF_{9-41} resulted in inhibition of c-Fos induction by nitrous oxide. Remarkable c-Fos inductions in the ependymal cells on the ventricular surface (*A–D*) represent the nonspecific effect of the intracerebroventricular injection through the chronic cannula. V = fourth ventricle. *Scale bar* = 50 μm.

as evaluated with the TFL test. Because the CRF antagonist had no effect on the antinociceptive action of dexmedetomidine (an α2 agonist that *inhibits* the LC neurons),[15] the action of CRF is selective for the antinociceptive action of nitrous oxide. These results suggest that nitrous oxide activates the CRF-containing neurons in the brain, which are causally related to its antinociceptive effect through the activation of noradrenergic systems.

Corticotropin-releasing factor is a key mediator of various stress responses. The exogenous administration of CRF produces behavioral activation and enhances behavioral responses to stressors.[5] Studies using CRF antagonists have provided evidence to suggest that brain CRF systems play an important role in mediating behavioral, endocrinologic, and physiologic responses to stressors.[5,16] Evidence indicates that CRF acts as a neurotransmitter; CRF receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways.[7] The CRF system reportedly enhances neuronal activity in the LC, the largest collection of noradrenergic neurons in the brain,[8,17] and increases norepinephrine release.[18,19] Furthermore, anatomical evidence indicates that the LC neurons are directly innervated by CRF neurons,[9] and intracerebroventricular or local administration of CRF activates the noradrenergic LC neurons.[20] In addition, a feed-forward interaction between CRF and noradrenergic systems has been postulated,[5] because noradrenergic innervation in the PVN further stimulates release of CRF.[21] Because the CRF system is activated under stressful conditions, a variety of stressors can activate the LC neurons through the CRF system.[22,23] Indeed, the CRF concentration in the LC is modulated by stress.[17,24] Central CRF neuronal systems are involved in mediating the stress-induced changes in the dynamics of brain noradrenergic systems in rats.[25] Furthermore, intracerebroventricular or direct administration of CRF antagonists prevented the stress-induced activation of the LC neurons[26]

It has also been reported that CRF produces antinociception[27]; for example, intracerebroventricular injection of CRF produces antinociception in the hot-plate test,[11] and although the precise mechanism is still unclear, several studies suggest that descending inhibitory mechanisms may be involved.[6,28] The antinociceptive effect of intracerebroventricular administration of CRF is antagonized by 6-hydroxydopamine and prazosin, thereby suggesting the involvement of the noradrenergic system in CRF-induced antinociception.[11] Considering that CRF activates the LC, the CRF system may mediate endogenous antinociception by triggering descending inhibitory pathways originating from the LC. This may also apply to stress-induced analgesia. Cold-water swim analgesia was inhibited by lesioning of the PVN,[29] a prominent site of CRF production. Furthermore, several studies indicate that acute stress-induced analgesia is

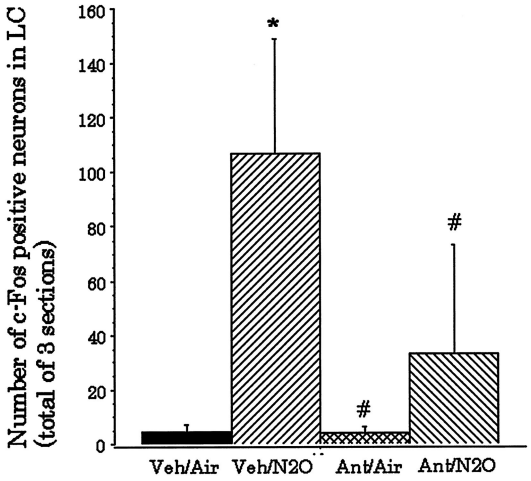

Fig. 4. Effect of corticotropin-releasing factor (CRF) antagonist, α-helical CRF_{9-41}, on nitrous oxide–induced c-Fos expression in the locus ceruleus (LC). Rats were pretreated with intracerebroventricular vehicle (Veh) or CRF antagonist (Ant) and exposed for 60 min to air or nitrous oxide. *Bars* represent total numbers of c-Fos–positive tyrosine hydroxylase neurons in the LC from three sections (mean ± SD). Nitrous oxide exposure induced remarkable c-Fos immunoreactivity in the LC (Veh/Air *vs.* Veh/N_2O). Induction of c-Fos immunoreactivity by nitrous oxide exposure was significantly suppressed by pretreatment with CRF antagonist (Veh/N_2O *vs.* Ant/N_2O). *$P < 0.0001$ *versus* vehicle/air, #$P < 0.0005$ *versus* vehicle/N_2O, n = 5–7 per group.

mediated *via* a noradrenergic system in animal models indicative of the interaction between the CRF and noradrenergic pathways in antinociception.[30,31]

The antinociceptive action of nitrous oxide has also been strongly linked to descending noradrenergic inhibitory pathways.[4] We previously demonstrated that nitrous oxide activated noradrenergic neurons in the LC and in other brainstem noradrenergic nuclei, and lesioning of these nuclei, which gives rise to the descending inhibitory pathways, inhibited the antinociceptive effect of nitrous oxide.[1] Also, we and others have shown that nitrous oxide increases norepinephrine release in the brain and the spinal cord.[2,3] Both antagonism of the adrenergic receptors[4] and depletion of norepinephrine[3] in the spinal cord inhibited the antinociceptive effect of nitrous oxide.

Although the mechanisms of LC activation by nitrous oxide have been unclear, the involvement of the CRF system in the brain is strongly suggested by the fact that exposure to nitrous oxide activated CRF containing neurons of the rat PVN and that intracerebroventricular administration of CRF antagonist inhibited the activation of the LC neurons by nitrous oxide. In addition, the antinociceptive action of nitrous oxide is also mediated, at least in part, by the CRF system in the brain because intracerebroventricular administration of CRF antagonist resulted in inhibition of nitrous oxide–induced antinociception but did not inhibit antinociception affected by dexmedetomidine. Taken together, we speculate that nitrous oxide activates the CRF system in the brain resulting in the stimulation of the LC and descending noradrenergic inhibitory pathways. Although this study focused on the activation of the LC, other noradrenergic nuclei in the brainstem, namely A5 and A7, could also be involved in the antinociceptive action of nitrous oxide. In fact, these nuclei originate descending noradrenergic inhibitory pathways that are activated by inhalation of nitrous oxide.[1]

Corticotropin-releasing factor induces transcription of proopiomelanocortin, which is a precursor of corticotropin, melanocyte-stimulating hormone, and β-endorphin. Furthermore, about 20% of CRF neurons in the PVN contain enkephalin.[32] Thus, activation of CRF system by nitrous oxide could lead to the antinociceptive action through opioidergic mechanisms. In fact, several studies have shown that opiate system could be involved in the antinociceptive effect of nitrous oxide.[4]

Although the mechanism underlying the activation of the CRF system by nitrous oxide is still unclear, it may result from the recently reported *N*-methyl-D-aspartate (NMDA)-antagonistic effect of nitrous oxide.[33,34] Nitrous oxide undergoes one electron reduction, and therefore is producing free-radical products.[35] The ionotropic NMDA receptor contains a redox-sensitive site, which might be a target for nitrous oxide.[36] The noncompetitive NMDA receptor antagonist MK801 activates CRF neurons in the rat PVN,[37] and some NMDA antagonists have been shown to exert antinociceptive effects at least partly through a supraspinal mechanism,[38,39] as has been shown in nitrous oxide antinociception. However, we should also note that the majority of studies using pure NMDA antagonists have failed to show antinociception to acute noxious stimuli.

Although animals were exposed to nitrous oxide as a sole anesthetic in our study, this gas is usually used in combination with other agents such as isoflurane or propofol in the clinical arena. The descending inhibitory mechanism of nitrous oxide antinociception can be inhibited by the concomitant use of general anesthetics because of the suppressive effect of these agents. In fact, our unpublished data showed that nitrous oxide–induced c-Fos expression in CRF neurons was dose-dependently inhibited by the concomitant use of isoflurane or propofol.[40,41] Evidence indicates that volatile anesthetics antagonize nitrous oxide antinociception in the rat[42]; therefore, nitrous oxide is a less effective analgesic in healthy volunteers coadministered sevoflurane.[43] How-

ever, our results do not refute the additive minimum alveolar concentration effects of gas anesthetics, because definition of minimum alveolar concentration is based on anesthetic, not analgesic, effects. Furthermore, our study does not exclude the possibility that mechanisms of nitrous oxide antinociception other than descending noradrenergic inhibition may exist.

Direct evidence of CRF release by nitrous oxide exposure is lacking in our study because the activation of CRF-containing neurons does not necessarily indicate that CRF secretion is enhanced. In fact, CRF-containing neurons are reported to contain other neuropeptides such as neurotensin and enkephalin.[32] However, the finding that the CRF antagonist inhibited both the activation of brainstem nuclei and the antinociceptive action of nitrous oxide indicates the direct involvement of CRF in these actions of nitrous oxide. Although there are several technical difficulties, direct demonstration of nitrous oxide-mediated CRF release in the brain would corroborate our findings.

In summary, we have used c-Fos expression as a marker of neuronal activation and demonstrated that nitrous oxide activates CRF-containing neurons in the PVN of the rat hypothalamus. Intracerebroventricular administration of CRF antagonist inhibited the activation of the LC by nitrous oxide. In addition, the CRF antagonist inhibited the antinociceptive action of nitrous oxide as measured by the tail-flick assay. These data indicate that the CRF system plays a pivotal role in the mechanism of nitrous oxide–induced acute antinociception.

References

1. 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
2. Karuri AR, Kugel G, Engelking LR, Kumar MS: Alterations in catecholamine turnover in specific regions of the rat brain following acute exposure to nitrous oxide. Brain Res Bull 1998; 45:557–61
3. Zhang C, Davies MF, Guo TZ, 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
4. Guo TZ, Poree L, Golden W, Stein J, Fujinaga M, Maze M: Antinociceptive response to nitrous oxide is mediated by supraspinal opiate and spinal alpha 2 adrenergic receptors in the rat. ANESTHESIOLOGY 1996; 85:846–52
5. Koob GF: Corticotropin-releasing factor, norepinephrine, and stress. Biol Psychiatry 1999; 46:1167–80
6. Lariviere WR, Melzack R: The role of corticotropin-releasing factor in pain and analgesia. Pain 2000; 84:1–12
7. Blank T, Nijholt I, Grammatopoulos DK, Randeva HS, Hillhouse EW, Spiess J: Corticotropin-releasing factor receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways in mouse hippocampus: Role in neuronal excitability and associative learning. J Neurosci 2003; 23:700–7
8. Curtis AL, Lechner SM, Pavcovich LA, Valentino RJ: Activation of the locus coeruleus noradrenergic system by intracoerulear microinfusion of corticotropin-releasing factor: Effects on discharge rate, cortical norepinephrine levels and cortical electroencephalographic activity. J Pharmacol Exp Ther 1997; 281: 163–72
9. Van Bockstaele EJ, Colago EE, Valentino RJ: Corticotropin-releasing factor-containing axon terminals synapse onto catecholamine dendrites and may presynaptically modulate other afferents in the rostral pole of the nucleus locus coeruleus in the rat brain. J Comp Neurol 1996; 364:523–534
10. Emoto H, Tanaka M, Koga C, Yokoo H, Tsuda A, Yoshida M: Corticotropin-releasing factor activates the noradrenergic neuron system in the rat brain. Pharmacol Biochem Behav 1993; 45:419–22
11. Bianchi M, Panerai AE: CRH and the noradrenergic system mediate the antinociceptive effect of central interleukin-1 alpha in the rat. Brain Res Bull 1995; 36:113–7
12. Harris JA: Using c-fos as a neural marker of pain. Brain Res Bull 1998; 45:1–8
13. Okumura T, Yamada H, Motomura W, Kohgo Y: Cocaine-amphetamine-regulated transcript (CART) acts in the central nervous system to inhibit gastric acid secretion via brain corticotropin-releasing factor system. Endocrinology 2000; 141:2854–60
14. Curtis AL, Grigoriadis DE, Page ME, Rivier J, Valentino RJ: Pharmacological comparison of two corticotropin-releasing factor antagonists: In vivo and in vitro studies. J Pharmacol Exp Ther 1994; 268:359–65
15. Nelson LE, Lu J, Guo T, Saper CB, Franks NP, Maze M: The alpha2-adrenoceptor agonist dexmedetomidine converges on an endogenous sleep-promoting pathway to exert its sedative effects. ANESTHESIOLOGY 2003; 98:428–36
16. Bakshi VP, Smith-Roe S, Newman SM, Grigoriadis DE, Kalin NH: Reduction of stress-induced behavior by antagonism of corticotropin-releasing hormone 2 (CRH2) receptors in lateral septum or CRH1 receptors in amygdala. J Neurosci 2002; 22:2926–35
17. Page ME, Abercrombie ED: Discrete local application of corticotropin-releasing factor increases locus coeruleus discharge and extracellular norepinephrine in rat hippocampus. Synapse 1999; 33:304–13
18. Schulz C, Lehnert H: Activation of noradrenergic neurons in the locus coeruleus by corticotropin-releasing factor: A microdialysis study. Neuroendocrinology 1996; 63:454–8
19. Smagin GN, Swiergiel AH, Dunn AJ: Corticotropin-releasing factor administered into the locus coeruleus, but not the parabrachial nucleus, stimulates norepinephrine release in the prefrontal cortex. Brain Res Bull 1995; 36:71–6
20. Valentino RJ, Foote SL: Corticotropin-releasing hormone increases tonic but not sensory-evoked activity of noradrenergic locus coeruleus neurons in unanesthetized rats. J Neurosci 1988; 8:1016–25
21. Alonso G, Szafarczyk A, Balmefrezol M, Assenmacher I: Immunocytochemical evidence for stimulatory control by the ventral noradrenergic bundle of parvocellular neurons of the paraventricular nucleus secreting corticotropin releasing hormone and vasopressin in rats. Brain Res 1986; 397:297–307
22. Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ: Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 1995; 64:477–505
23. Pezzone MA, Lee WS, Hoffman GE, Pezzone KM, Rabin BS: Activation of brainstem catecholaminergic neurons by conditioned and unconditioned aversive stimuli as revealed by c-Fos immunoreactivity. Brain Res 1993; 608:310–8
24. Chappell PB, Smith MA, Kilts CD, Bissette G, Ritchie J, Anderson C, Nemeroff CB: Alterations in corticotropin-releasing factor-like immunoreactivity in discrete rat brain regions after acute and chronic stress. J Neurosci 1986; 6:2908–14
25. Li J, Takeda H, Tsuji M, Liu L, Matsumiya T: Antagonism of central CRF systems mediates stress-induced changes in noradrenaline and serotonin turnover in rat brain regions. Methods Find Exp Clin Pharmacol 1998; 20:409–17
26. Page ME, Valentino RJ: Locus coeruleus activation by physiological challenges. Brain Res Bull 1994; 35:557–60
27. Hargreaves KM, Mueller GP, Dubner R, Goldstein D, Dionne RA: Corticotropin-releasing factor (CRF) produces analgesia in humans and rats. Brain Res 1987; 422:154–7
28. Bellchambers CE, Chieng B, Keay KA, Christie MJ: Swim-stress but not opioid withdrawal increases expression of c-fos immunoreactivity in rat periaqueductal gray neurons which project to the rostral ventromedial medulla. Neuroscience 1998; 83:517–24
29. Truesdell LS, Bodnar RJ: Reduction in cold-water swim analgesia following hypothalamic paraventricular nucleus lesions. Physiol Behav 1987; 39:727–31
30. Oluyomi AO, Hart SL: Alpha-adrenoceptor involvement in swim stress-induced antinociception in the mouse. J Pharm Pharmacol 1990; 42:778–84
31. Kaada B: Neurotransmitters in "the smoke reflex" in rabbits. Gen Pharmacol 1987; 18:61–8
32. Ceccatelli S, Eriksson M, Hokfelt T: Distribution and coexistence of corticotropin-releasing factor-, neurotensin-, enkephalin-, cholecystokinin-, galanin- and vasoactive intestinal polypeptide/peptide histidine isoleucine-like peptides in the parvocellular part of the paraventricular nucleus. Neuroendocrinology 1989; 49:309–23
33. Jevtovic-Todorovic V, Todorovic SM, Mennerick S, Powell S, Dikranian K, Benshoff N, Zorumski CF, Olney JW: Nitrous oxide (laughing gas) is an NMDA antagonist, neuroprotectant and neurotoxin. Nat Med 1998; 4:460–3
34. Yamakura T, Harris RA: Effects of gaseous anesthetics nitrous oxide and xenon on ligand-gated ion channels: Comparison with isoflurane and ethanol. ANESTHESIOLOGY 2000; 93:1095–101
35. Chinev S, Bakalova R, Peneva V, Uzunova P, Galabova T, Sokolova Z, Ribarov S: Nitrous oxide with fentanyl and droperidol minimizes lipid peroxidation in the liver. Eur J Anaesthesiol 1995; 12:155–62
36. Lipton S, Choi Y, Pan Z, Lei S, Chen H, Sucher N, Loscalzo J, Singel D, Stamler J: A redox-based mechanism for the neuroprotective and neurodestruc-

tive effects of nitric oxide and related nitroso-compounds. Nature 1993; 364: 626–32

37. Lee S, Rivier C, Torres G: Induction of c-fos and CRF mRNA by MK-801 in the parvocellular paraventricular nucleus of the rat hypothalamus. Brain Res Mol Brain Res 1994; 24:192–8

38. Forman LJ: NMDA receptor antagonism produces antinociception which is partially mediated by brain opioids and dopamine. Life Sci 1999; 64:1877–87

39. Chizh BA, Reissmuller E, Schlutz H, Scheede M, Haase G, Englberger W: Supraspinal vs spinal sites of the antinociceptive action of the subtype-selective NMDA antagonist ifenprodil. Neuropharmacology 2001; 40:212–20

40. Sawamura S, Obara M, Tamai H, Ito N, Hanaoka K: Isoflurane suppresses the nitrous oxide-induced activation of corticotropin releasing factor-containing neurons in rat paraventricular nucleus of hypothalamus (abstract). ANESTHESIOLOGY 2001; 95:A718

41. Obara M, Sawamura S, Hagihara R, Hanaoka K: Propofol inhibits nitrous oxide-induced activation of corticotropin releasing factor-containing neurons in rat paraventricular nucleus of hypothalamus (abstract). ANESTHESIOLOGY 2001; 95:A714

42. Goto T, Marota JJ, Crosby G: Volatile anaesthetics antagonize nitrous oxide and morphine-induced analgesia in the rat. Br J Anaesth 1996; 76:702–6

43. Janiszewski DJ, Galinkin JL, Klock PA, Coalson DW, Pardo H, Zacny JP: The effects of subanesthetic concentrations of sevoflurane and nitrous oxide, alone and in combination, on analgesia, mood, and psychomotor performance in healthy volunteers. Anesth Analg 1999; 88:1149–54