The Analgesic Action of Nitrous Oxide Is Dependent on the Release of Norepinephrine in the Dorsal Horn of the Spinal Cord

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Background: The authors and others have demonstrated that supraspinal opiate receptors and spinal α2 adrenoceptors are involved in the analgesic mechanism for nitrous oxide (N2O). The authors hypothesize that activation of opiate receptors in the periaqueductal gray results in the activation of a descending noradrenergic pathway that releases norepinephrine onto α2 adrenoceptors in the dorsal horn of the spinal cord.

Methods: The spinal cord was transected at the level of T3–T4 in rats and the analgesic response to 70% N2O in oxygen was determined by the tail flick latency test. In a separate experiment in rats a dialysis fiber was positioned transversely in the dorsal horn of the spinal cord at the T12 level. The following day, the dialysis fiber was infused with artificial cerebrospinal fluid at a rate of 1.3 μl/min, and the effluent was sampled at 30-min intervals. After a 60-min equilibration period, the animals were exposed to 70% N2O in oxygen. The dialysis experiment was repeated in animals that were pretreated with naltrindone (10 mg/kg, intraperitoneally) before N2O. In a third series, spinal norepinephrine was depleted with n-(2-chloroethyl)-n-ethyl-2-bromobenzylamine (DSP-4), and the analgesic response to 70% N2O in oxygen was determined.

Results: The analgesic effect of N2O was prevented by spinal cord transection. After exposure to N2O, there was a fourfold increase in norepinephrine release in the first 30-min period, and norepinephrine was still significantly elevated after 1 h of exposure. The increased norepinephrine release was prevented by previous administration of naltrindone. Depletion of norepinephrine in the spinal cord blocked the analgesic response to N2O.

Conclusions: A descending noradrenergic pathway in the spinal cord links N2O-induced activation of opiate receptors in the periaqueductal gray, with activation of α2 adrenoceptors in the spinal cord. N2O-induced release of norepinephrine in the dorsal horn of the spinal cord is blocked by naltrindone, as is the analgesic response. Spinal norepinephrine is necessary for the analgesic response to N2O. (Key words: Analgesia; descending; inhibition; naltrindone; nitrous oxide; norepinephrine.)

Nitrous oxide (N2O) is one of the most common agents used in anesthetic practice. Although its first use as an analgesic was described more than 150 yr ago, its mechanism of action has not been defined. Many lines of evidence indicate that N2O has its analgesic action by activating discrete neuronal pathways. Recently, we reported that α2 adrenoceptors in the spinal cord were necessary to transduce the acute antinociceptive response to N2O. A mechanism of action for N2O also has been proposed in which opiate receptors are activated through the release of endogenous opiate ligands.8,9 The periaqueductal gray (PAG) has long been known to be an important site for the analgesic action of opiates (cf.6). We7 and others8 demonstrated that the analgesic properties of N2O could be blocked by the discrete introduction of opiate antagonists directly into the PAG.

Several enigmatic issues concerning N2O remain, including the circuitry involved between the opiate receptors in the PAG and the α2 adrenoceptors in the spinal cord. Clearly, if an adrenergic receptor mediates the action in the spinal cord, then an endogenous pathway that releases norepinephrine or epinephrine should be activated by N2O exposure. Further evidence for the involvement of a descending noradrenergic pathway in the action of N2O could be acquired by determining whether its disruption, either by transection or by local administration of the neurotoxin n-(2-chloroethyl)-n-ethyl-2-bromobenzylamine (DSP-4), could eliminate N2O analgesic action.
In this series of studies we sought to answer the following questions:

1. Does a pathway descend in the spinal cord to link N\textsubscript{2}O-induced activation of opiate receptors in the PAG with activation of \(\alpha\) adrenoceptors in the spinal cord?
2. Does the descending pathway release norepinephrine in the dorsal horn in the spinal cord in response to N\textsubscript{2}O?
3. Is release of norepinephrine blocked by strategies that prevent N\textsubscript{2}O-induced analgesia?
4. Is spinal norepinephrine necessary for the analgesic response to N\textsubscript{2}O?

**Methods**

The experimental protocol was approved by the Animal Care and Use Committee at the Palo Alto Veterans Administration Medical Center. Male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA) weighing 250-380 g were anesthetized with isoflurane and the lateral surfaces of vertebra T13 were exposed. Bilateral holes were made through the bone to expose the spinal cord at the level of the dorsal horn.

Except for a 2-mm dialysis zone, dialysis fibers (diameter 200 mm; molecular weight cutoff = 9,000; Spectrum Laboratories, Laguna Hills, CA) were coated with a thin layer of silicon rubber. One end of the fiber was connected to a 90° angled stainless tubing made from a 22-gauge stainless steel needle. A stainless steel dissecting pin was affixed to the lumen of the other end of the fiber. By pushing the pin through the spinal cord and pulling it out the other side, the fiber was positioned so that the uncoated portion of the fiber was located within the dorsal horn of the spinal cord.

To verify that the fiber traversed the dorsal horn, the spinal cord was removed and fixed in 10% formalin for histologic confirmation of cannula placement. Only animals with the cannula located below lamina I and above the central canal were included in the study.

**Histologic Examination**

Rats were anesthetized with halothane, and laminec-tomy was performed at the T3–T4 level. Spinal pro-
cesses and laminae were removed to expose a circular region of dura. The dura was opened and the spinal cord was severed at the T3-T4 level. The muscles were sutured over the laminectomy site and the skin was closed with wound clips. The animals were exposed to N₂O and tested for tail flick response within 6 h after surgery.

**Intrathecal Administration of DSP-4**

Rats were anesthetized with isoflurane, an incision was made over the cervical spine, and a small puncture was made in the dura mater. PE-10 polyethylene tubing (0.28 mm ID) was threaded 8.5 cm into the intrathecal space so the tip of the catheter was positioned at the lumbar level. This tubing was then sutured in place, and the skin was sutured over the tubing. After allowing 7 days for recovery, DSP-4 (100 or 300 µg) was administered in 10 µl normal saline using a perfusion pump at a rate of 10 µl/min followed by a 10-µl flush of normal saline. Behavioral testing or killing for the determination of spinal norepinephrine levels was performed 10 days later.

The levels of norepinephrine in the lumbar enlargement of spinal cord were measured using the high-performance liquid chromatography. Rats were exposed to 100% carbon dioxide for 35 s and then killed. The spinal cord was rapidly extruded from the spinal canal using ice-cold saline; the lumbar enlargement was isolated and weighed. The tissue was put into 600 µl perchloric acid, 2%, and 2 × 10⁻⁶ M dihydroxybenzylamine, and homogenized and centrifuged at 1,200g for 15 min at 4°C. The supernatant was removed and stored at -80°C for later analysis.

**Nociceptive Testing Procedures**

Nociception was assessed by the tail flick response to a noxious thermal stimulus, as previously described. In brief, a high-intensity light beam was focused on the tail, and the time for the rat to move its tail out of the light was recorded as tail flick latency. The latency from three sites on the tail were averaged and a cut-off time of 10 s was predetermined to prevent tissue damage. Baseline measurements consisted of a set of three tail flick determinations at 2-min intervals. Baseline tail flick latencies ranged between 3 and 4 s. In some cases, percent maximal possible effect (%MPE) was calculated as

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\frac{\text{latency} - \text{baseline}}{\text{(cut-off time} - \text{baseline})} \times 100
\]

**Gas Exposures**

All gas exposures were performed in a clear plastic chamber (92 × 48 × 38 cm) with a sliding door on one side (for insertion of the rats). This airtight chamber was large enough to contain the infusion pump and the analgesimeter device. Fresh test gases (10 l/min) were introduced into the chamber via an inflow port, circulated throughout the chamber by a small fan, and purged by vacuum set to aspirate at the same rate as the fresh gas inflow. Oxygen concentration in the chamber was maintained between 22-30%, while N₂O concentration was maintained at 0 or 70% by adjusting the flow rates of N₂O, air, and nitrogen (Liquid Carbonic, Houston, TX). Gas concentrations were measured continuously and flow rates were adjusted appropriately to maintain the desired concentrations.

**Statistics**

Release data were analyzed by analysis of variance for repeated measures and *a posteriori* by Scheffé or Bonferroni tests. Nociceptive data were analyzed by unpaired Student *t* test or analysis of variance for repeated measures and *a posteriori* by the Bonferroni multiple comparisons test when appropriate.

**Results**

**Spinal Cord Transection Blocked the Analgesic Action of Nitrous Oxide**

Spinal transection at level T3-T4 did not affect the baseline tail flick latency but did block the analgesic action of 70% N₂O (fig. 1).

**Nitrous Oxide Stimulated the Spinal Release of Norepinephrine**

Nitrous oxide caused approximately a fourfold increase in norepinephrine release within the first 30-min collection period (fig. 2). This level of release decreased in subsequent collection periods, reaching baseline values during the 60- to 90-min collection period.

**Naltrexone Suppressed Spinal Norepinephrine Release**

Systemic opiate antagonists, such as naloxone, block the analgesic action of N₂O possibly by antagonizing the action of endogenously released opiates at the level of the PAG. To determine whether an opiate antagonist also suppressed norepinephrine release evoked by N₂O, the levels of spinal norepinephrine release were mea-
Fig. 1. Spinal cord transection blocked the analgesic action of N2O. The spinal cords of rats were sectioned at the T3–T4 level before exposure to 70% N2O. The analgesia was measured after 30 min of N2O exposure. (A) Tail flick latencies before and after N2O in sham and transected animals. Spinal transection did not affect baseline tail flick latencies but did abolish N2O analgesia. Data are expressed as mean ± SEM. ***P < 0.001 Bonferroni multiple comparison test (n = 9). (B) The percent maximal possible effect of N2O was reduced in transected animals. Data are expressed as mean ± SEM. ***P < 0.001 unpaired t test (n = 9).

Fig. 2. N2O caused norepinephrine release in the dorsal horn of the rat spinal cord. In rats, a dialysis fiber was placed at T12 during isoflurane anesthesia. The dialysis tubing was stabilized with dental acrylic and the ends were externalized. The following day, the inflow of the dialysis tubing was connected via a swivel to a pump, which infused artificial cerebrospinal fluid at a rate of 1.3 μl/min. Microdialysate was collected during a 60-min equilibration period, with the rats breathing air, then the rats were exposed to 70% N2O for 90 min. The effluent was sampled at 30-min intervals with a fraction collector. Norepinephrine was assayed by high-performance liquid chromatography with electrochemical detection. The position of the dialysis portion of the fiber was confirmed histologically at the conclusion of the experiment. Data were analyzed by analysis of variance for repeated measures and a posteriori by Scheffé test. Data are expressed as mean ± SEM. *P < 0.05 (n = 9).

Discussion

This study shows that N2O causes the release of norepinephrine in the spinal cord in the awake freely moving rat. This release decreases with continued N2O exposure and is dependent on the presence of a functional opiate receptor. In keeping with the hypothesis that N2O analgesia is mediated by the release of norepinephrine, depletion of norepinephrine stores by DSP-4 or elimination of descending noradrenergic transmission by spinal cord transection attenuates N2O analgesia. Although spinal cord transection and the insertion of the microdialysis fiber may cause a degree of nerve injury that could modify norepinephrine release characteristics, all effects were temporally related to the onset of N2O exposure. Although DSP-4 treatment is known to also deplete serotonin,14 the lack of analgesic effect of...
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Fig. 3. Block of opiate receptors with naltrexone suppressed N₂O-evoked norepinephrine release. (A) In the absence of naltrexone (saline), a 30-min exposure to 70% N₂O had an analgesic action. Pretreatment with naltrexone (10 mg/kg intraperitoneal) given 1 and 3 h before the tail flick test blocked the analgesic effect of 70% N₂O. *P < 0.05 (n = 6–12). (B) Naltrexone (10 mg/kg) was administered 90 min before a 30-min exposure to 70% N₂O. Data are expressed as mean ± SEM. **P < 0.001 (n = 6–9).

N₂O in DSP-4-treated animals coupled with the knowledge that spinal α₂ adrenoceptors are necessary for N₂O analgesia indicates that the noradrenergic system is necessary for N₂O analgesia.

By measuring neurotransmitter turnover, others have found that N₂O stimulates norepinephrine turnover in various brain regions.¹⁵ N₂O also has been found to increase brain dopamine turnover,¹⁶ although when turnover in discrete regions of the brain was evaluated, N₂O caused a decreased turnover rate of dopamine in the hippocampus and striatum but an increase in the olfactory bulb.¹⁵ These studies indicate that N₂O causes region-specific alterations in steady state levels and turnover rates of dopamine and norepinephrine within the central nervous system. In addition, N₂O suppression of the activity of wide-dynamic-range neurons, in which activity has been linked to pain transmission, depends on an intact descending inhibitory pathway.¹⁷

The mechanism by which N₂O affects norepinephrine release is not clear, although current evidence supports a pivotal role for release of endogenous opiate peptides. Previously, we found that an opiate antagonist administered into the PAG reduced the analgesic action of N₂O.¹⁴ Coupled with the current finding that N₂O-evoked norepinephrine release is blocked by naltrexone, these data are consistent with the hypothesis that N₂O causes the release of endogenous opiate peptides in the PAG, as has been observed by others. Candidate peptides known to be present in the PAG are enkephalin, β-endorphins, or dynorphin.¹⁸ There is some evidence that β-endorphins may be involved because their release is stimulated by N₂O both in vivo and in vitro.¹⁸ Antiserum against β-endorphin but not

Fig. 4. Depletion of spinal norepinephrine with DSP-4-blocked N₂O analgesia. (A) Intrathecal administration of DSP-4 (300 µg in a volume of 10 µl) 10 days before killing reduced the levels of norepinephrine in the spinal cord. ***P < 0.001 (n = 6–7). (B, C) DSP-4, (100 or 300 µg in a volume of 10 µl) was intrathecally administered 10 days before testing the analgesic action of a 30-min exposure to 70% N₂O. There was no change in baseline tail flick latencies with DSP-4 treatment (B), but the analgesic action of N₂O was reduced (C). Data are expressed as mean ± SEM. *P < 0.05, ***P < 0.001 (n = 7 or 8).

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against metenkephalin also blocked the \( \text{N}_2\text{O} \)-induced antinociception in rats in the hot plate test.\(^{19}\) However, other studies indicate that metenkephalin may be involved in the analgesic effect of \( \text{N}_2\text{O} \) because cerebrospinal fluid levels of metenkephalin taken from the third ventricle of awake dogs increased significantly; no changes were noted in concentrations of dynorphin A, dynorphin B, or \( \beta \)-endorphin.\(^{5}\) \( \text{N}_2\text{O} \) also caused an increase in metenkephalin-like immunoreactivity in the brain stem, spinal cord, hypothalamus, and corpus striatum in rats.\(^{20}\) Enkephalin- and dynorphin-containing cells are present in the PAG but are also found in many other areas of the brain;\(^{18,21,22}\) therefore, \( \text{N}_2\text{O} \) may have an action in brain regions rostral to the PAG. It is known that analgesia can be evoked by electrical stimulation of various sites in the brain, such as the habenular complex,\(^{22,23}\) arcuate nucleus,\(^{24}\) and amygdala.\(^{25}\) Similar to \( \text{N}_2\text{O} \) analgesia, this analgesia is sensitive to blockade by opiate \(^{26-28}\) and \( \alpha \)-adrenergic antagonists.\(^{23,24}\) Further work is necessary to elucidate the identity of the opiate peptides and mechanism by which \( \text{N}_2\text{O} \) causes their release.

Several lines of evidence indicate that spinally projecting noradrenergic neurons mediate the antinociception produced by the activation of the PAG. Intrathecal injection of \( \alpha_2 \)-adrenergic antagonists can reduce the antinociception produced by either electrical\(^{26}\) or chemical\(^{27}\) stimulation of PAG neurons. Intrathecal injection of an \( \alpha_2 \) antagonist can also attenuate the antinociception produced by microinjection of morphine in the PAG.\(^{28}\) Additionally, discrete injection of morphine into the PAG produces an increase in norepinephrine metabolites in the spinal cord, and its analgesic effects are attenuated by previous depletion of norepinephrine stores in the spinal cord.\(^{29}\) Electrophysiologic studies also showed that \( \alpha_2 \) adrenoceptors in the spinal cord contribute to the mediation of the PAG-induced inhibition of dorsal horn cell activity.\(^{31,30}\)

There are three nuclei from which noradrenergic neuronal projections to the spinal cord originate. Using the tract tracing methods and combinations of lesions with histochemical methods, numerous studies have shown that noradrenergic neuronal projection to the spinal cord originates from the A5, A6 (locus coeruleus, subcoeruleus), and A7 cell groups.\(^{31,32}\) In the same substrain of animals used in this study, Basbaum's\(^{33}\) laboratory showed that the locus coeruleus is the major source of noradrenergic fibers to the dorsal horn region. All these nuclei receive projections from the PAG.\(^{34-37}\) Subsequent studies will seek to identify which noradrenergic nucleus is responsible for the analgesic action of \( \text{N}_2\text{O} \).

We have not conclusively established whether \( \text{N}_2\text{O} \)-induced norepinephrine release is mediated by activation of the descending noradrenergic pathways at a supraspinal or spinal site. In the context of our previous studies that the analgesic response to \( \text{N}_2\text{O} \) is caused by activation of opiate receptors in the PAG,\(^{7}\) and our current finding that naltrexone blocks \( \text{N}_2\text{O} \)-induced norepinephrine release in the dorsal horn of the spinal cord, we believe that the weight of evidence supports a supraspinal site of activation.

There is clear evidence that the acute antinociceptive properties of \( \text{N}_2\text{O} \) decrease over a relatively short time during continuous administration.\(^{38-41}\) The results of this study show that the release of norepinephrine also diminishes over roughly the same time span, pointing to a site for tolerance proximal to norepinephrine release. Interestingly, the ability of \( \text{N}_2\text{O} \) to affect dopamine turnover also diminishes progressively.\(^{10}\) However, the precise site of tolerance is not known. Prolonged exposure (18 h) to \( \text{N}_2\text{O} \) decreases opiate receptor density in rat brain stem,\(^{42}\) and this would be consistent with increased release of endogenous opiate peptides causing a down-regulation of the receptor system. This raises the possibility that, depending on the mechanism involved, "cross-tolerance" may develop to either exogenous opiate or \( \alpha_2 \)-agonist administration, or both, after \( \text{N}_2\text{O} \) exposure. Knowledge of the mechanism for \( \text{N}_2\text{O} \) tolerance may help to identify people who might be less sensitive to the analgesic action of \( \text{N}_2\text{O} \), and also allow the design of strategies to mitigate the development of tolerance to prolong the analgesic effect of \( \text{N}_2\text{O} \).

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