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Antinociceptive Response to Nitrous Oxide Is Mediated by Supraspinal Opiate and Spinal α_2 Adrenergic Receptors in the Rat

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Background: **Despite nearly 150 years of clinical use, the mechanism(s) of action of nitrous oxide (N_2O) remains in doubt. In some but not all studies the analgesic properties of N_2O can be attenuated by opiate receptor antagonists. The purported mechanism for the opiate antagonistic effect relates to the finding that N_2O increases supraspinal levels of endogenous opiates, although this finding has been disputed. Based on the observations that (1) N_2O promotes the release of catecholamines, including the endogenous α_2 adrenergic agonist norepinephrine, and (2) that descending noradrenergic inhibitory pathways are activated by opioid analgesics, this study sought to determine whether α_2 adrenergic receptors are involved in the antinociceptive action of nitrous oxide.**

Methods: **Institutional approval was obtained for the study. Rats breathed 70% N_2O and 30% O_2 in an enclosed chamber. After a 30-min exposure, significant antinociception was indicated by an increase in the latency response to a noxious stimulus (tail-flick latency). The tail-flick latency was tested in rats exposed to 70% N_2O after either systemic or regional (intrathecal or intracerebroventricular) injections with either competitive (atipamezole; yohimbine) or noncompetitive (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) α_2 adrenoceptor antagonists, or the opiate receptor antagonist naloxone.**

Results: **When administered systemically, both the opiate (naloxone) and α_2 adrenoceptor antagonists (atipamezole, yohimbine, and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) blocked the enhanced tail-flick latency response to N_2O. Naloxone administered intracerebroventricularly, but not intrathecally, blocked the enhanced tail-flick latency response to N_2O. Conversely, atipamezole administered intrathecally, but not intracerebroventricularly, blocked the enhanced tail-flick latency response to N_2O.**

Conclusions: **These data suggest that both supraspinal opiate and spinal α_2 adrenoceptors play a mediating role in the antinociceptive response to N_2O in rats. A possible mechanism may involve a descending inhibitory noradrenergic pathway that may be activated by opiate receptors in the periaqueductal gray region of the brain stem in the rat after exposure to N_2O. (Key words: Analgesic. Alpha 2. Nitrous oxide receptors. Opiate adrenergic receptors.)**

DESPITE nearly 150 years of clinical use, the mechanism(s) of action of nitrous oxide (N_2O) is still unknown. The reason for the paucity of information on its mechanism of action stems from the technical difficulties associated with studying a gas with such low potency (its anesthetic action can only be tested in a hyperbaric chamber because its median effective dose (ED_{50}) for anesthesia exceeds 100% v/v at 1 atm). Conversely, its analgesic action can be demonstrated at much lower concentrations ($\pm$20% at 1 atm in humans), a fact that lends itself to investigation of its analgesic (or antinociceptive in nonhuman animals in which the subjective "sensation" of pain cannot be measured) properties.

The mechanistic role of the opioidergic system for the analgesic properties of N_2O has been investigated with conflicting results. Almost 20 years ago, it was shown that the opiate antagonist, naloxone, antagonized the analgesic effect of N_2O in mice[1] and subsequently in humans,[2] which led the authors to speculate that opiate receptors mediated the analgesic effect of N_2O. This notion gained support from neurochemical studies that showed that plasma β endorphin concentrations and [Met^5]enkephalin[3] concentrations in the cerebrospinal fluid increase after N_2O exposure. However, other investigators could not corroborate the findings in [Met^5]enkephalin[4] or in the endorphins.[5,6] "Cross-tolerance" studies, in which the antinociceptive response to each of opioids or nitrous oxide is

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examined after prolonged administration of the other compound, have yielded confusing results. For example, after long-term administration of nitrous oxide, rats are still sensitive to the antinociceptive effect of systemically administered morphine, whereas the analgesic response to N_2O is lost in mice made tolerant to morphine. Therefore, in addition to the putative role of the opioidergic system, another neurotransmitter system, possibly a noradrenergic one, may also be involved.

The mechanism whereby stimulation of opiate receptors in supraspinal regions produces analgesia has been extensively investigated and appears, in part, to involve a descending noradrenergic pathway. Antinociception produced by supraspinally injected morphine is blocked by intrathecally administered antagonists of adrenergic receptors,[7,8] whereas it is enhanced by the intrathecal administration of drugs that block the reuptake of norepinephrine.[9] In addition, discrete injection of morphine into the periaqueductal gray increases norepinephrine metabolites in the spinal cord,[10] and the analgesic effect of morphine, administered into the periaqueductal gray, is attenuated by prior depletion of norepinephrine stores in the spinal cord.[11] These studies further suggest a role for noradrenergic mechanisms in the analgesic action of opioids, both endogenous and exogenous. In the current study, we investigated the role of opiate and α_2 adrenoceptors, at both spinal and supraspinal sites, in the antinociceptive effects of nitrous oxide in rats.

Methods

The experimental protocol was approved by the Animal Care and Use Committee at the Palo Alto Veterans Administration Medical Center. One hundred fifty male Sprague-Dawley rats (Bantin and Kingman, Fremont, CA) weighing 250 to 380 g were used. All tests were performed between 9 A.M. and 4 P.M. Each animal was used for only one set of studies to eliminate possible interaction between different doses and routes of drug administration.

Intracerebroventricular Administration of Drugs

To perform intracerebroventricular administration of opiate and α_2-adrenoceptor antagonists, a guide cannula was placed in the intraventricular space (lateral ventricle) in some rats. The animals were anesthetized with isoflurane and placed in the stereotactic frame. The guide cannula was placed using the following coordinates: 1 mm posterior to Bregma, 1 mm lateral, and 4 mm ventral to the skull surface. To inject the drug, a 30-gauge needle connected to polyethylene tubing was placed through the cannula, with its tip positioned 1 mm beyond the tip of the cannula. Naloxone 5, 10 μg and atipamezole 28, 70 μg, each dose in 10 μl, were injected using an infusion pump (Harvard Apparatus Inc., South Natick, MA) at a rate of 10 μl/min.

Intrathecal and Systemic Administration of Drugs

For intrathecal administration of the same compounds as were administered intracerebroventricularly, animals 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 internal diameter) was threaded 8.5 cm into the intrathecal space so that the tip of the catheter was positioned at the lumbar level. This tubing was then sutured in place, and the skin was sutured together over the tubing. After the appropriate recovery time of 4 to 6 days, the desired agents — atipamezole 7, 14 μg, naloxone 10, 100 μg, each dose in 10 μl — were injected through the intrathecal catheter using an infusion pump at a rate of 10 μl min^{-1}; thereafter the catheter was flushed with 10 μl 0.9% normal saline. For systemic administration of naloxone, atipamezole, and yohimbine (each 1 mg kg^{-1}) each was given *via* the intraperitoneal route, whereas 1 mg kg^{-1} N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was injected subcutaneously.

Antinociceptive Testing

The antinociceptive response was assessed using an analgesiometer to measure the tail-flick latency response. A high-intensity light was focused on the rat's tail and the time for the rat to move its tail out of the light beam was automatically recorded (tail-flick apparatus, Columbus Instruments, Columbus, OH) and referred to as tail-flick latency. A different patch of the tail was exposed to the light beam on each trial to minimize the risk of tissue damage. The animals were placed on the heating blanket to maintain the body and tail temperature during the experiment. A cut-off time of 10 sec was predetermined, at which time the trial was terminated if no response was observed. Each tail flick latency data point consisted of a mean of three trials for each animal.

Gas Exposures

All gas exposures were performed in a plexiglass chamber (36 inches long, 19 inches wide, and 15

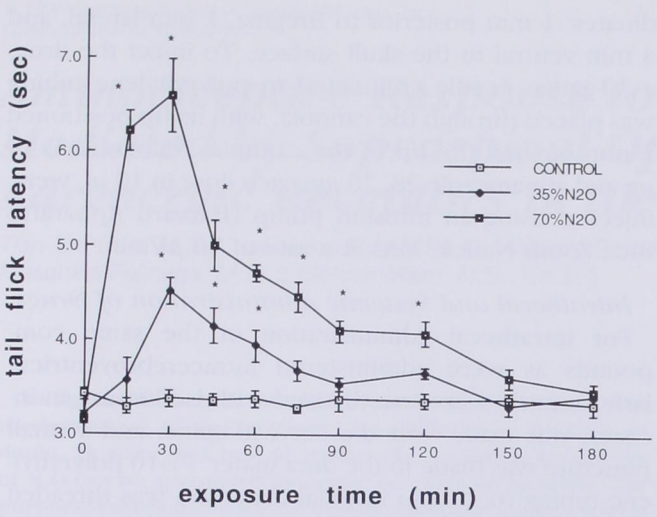

Fig. 1. Time course of the antinociceptive effect of nitrous oxide. Rats (n = 8 per group) were exposed to nitrous oxide (either 35% or 70%) or air in an exposure chamber. At designated times, the latency to eliciting a tail flick in response to a heat stimulus was measured.

Data are expressed as mean ± SEM.

*** = significantly different ($P < 0.05$) from baseline measurement.**

inches high) with a sliding door on one side (for insertion of the rats). This airtight chamber was large enough to contain the injection pump and the analgesiometer device. Fresh test gases (10 l/min) were introduced into the chamber *via* an inflow port, circulated throughout the chamber using 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% and 30%, whereas nitrous oxide concentration varied from 0, 35%, and 70% by adjusting the flow rates of nitrous oxide, air. and nitrogen (liquid carbonic). Gas concentrations were measured continuously and flow rates adjusted appropriately to maintain the desired concentrations.

Drug Administrations

Naloxone, yohimbine (Sigma Chemical Co., St Louis, MO), and atipamezole (Orion Farmos, Turku, Finland) dissolved in 0.9% normal saline were administered intraperitoneally 30 min before antinociceptive testing; For intracerebroventricular and intrathecal administrations, drugs were given 10 min before testing. EEDQ (Sigma Chemical Co.) was dissolved in 10% ethanol and injected subcutaneously 24 h before testing. All tests as well as intracerebroventricular and intrathecal injections were performed in the chamber. The reasons for using three α_2-adrenoceptor antagonists (atipamezole, yohimbine, and EEDQ) were as follows. Although atipamezole only has antagonist activity at the α_2-adrenoceptor,[12] it possesses an imidazole that facilitates binding to the imidazoline-preferring binding site, which may exert some physiologic effects.[13] Yohimbine, an indolealkylamine alkaloid, has no activity at the imidazoline-preferring binding site but does activate serotonin receptors nonspecifically.[14] EEDQ is a noncompetitive alkylating agent that also has activity at the dopamine receptor.[15]

Statistical Analysis

Results were analyzed using factorial analysis of variance and expressed as a mean ± SEM.

Results

Nitrous oxide produced a dose-dependent antinociceptive action as reflected by a change in tail-flick latency response (fig. 1). This effect is stable between 15 and 30 min of nitrous oxide exposure, after which

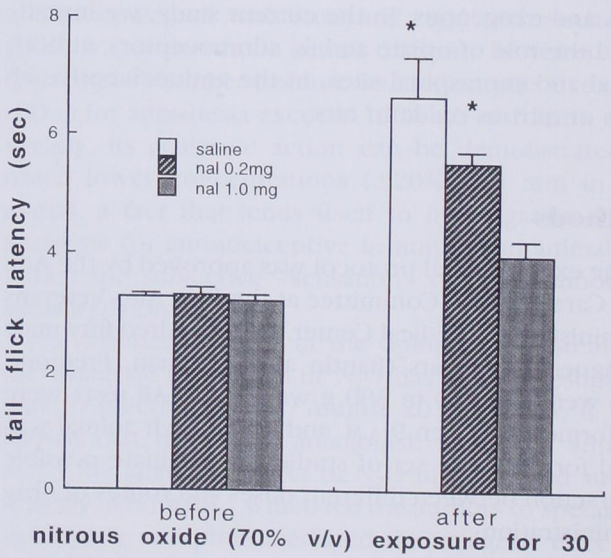

Fig. 2. Effect of naloxone on antinociceptive response to nitrous oxide. Three cohorts of rats (n = 6 per group) were given saline or naloxone, 0.2 or 1.0 mg kg^{-1}, administered intraperitoneally 30 min before the latency to elicit a tail flick in response to a heat stimulus was measured. Subsequently, the animals were exposed to nitrous oxide (70% v/v) for 30 min and the tail-flick latency measurement was repeated.

Data are expressed as mean ± SEM.

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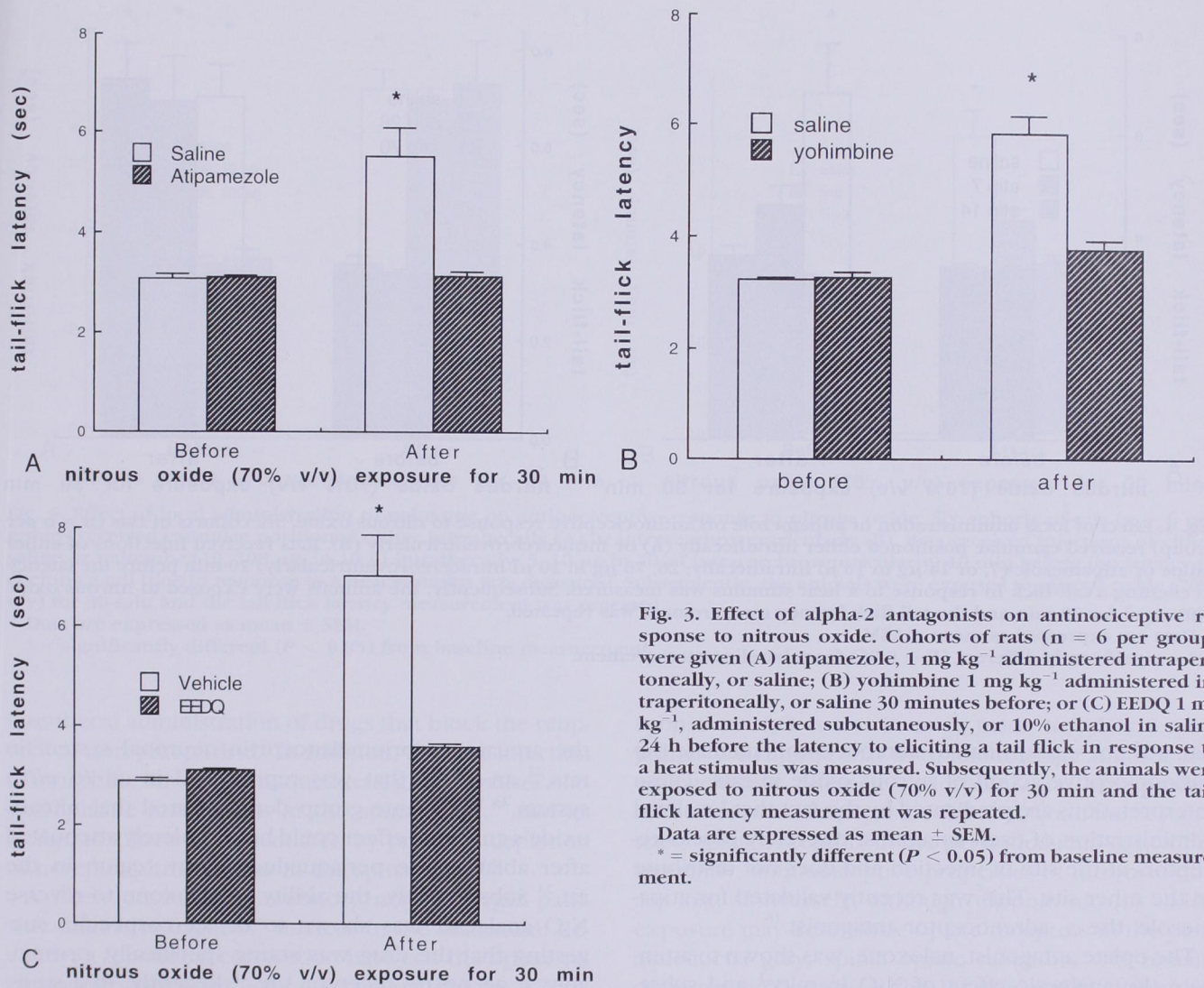

Fig. 3. Effect of alpha-2 antagonists on antinociceptive response to nitrous oxide. Cohorts of rats (n = 6 per group) were given (A) atipamezole, 1 mg kg^{-1} administered intraperitoneally, or saline; (B) yohimbine 1 mg kg^{-1} administered intraperitoneally, or saline 30 minutes before; or (C) EEDQ 1 mg kg^{-1}, administered subcutaneously, or 10% ethanol in saline 24 h before the latency to eliciting a tail flick in response to a heat stimulus was measured. Subsequently, the animals were exposed to nitrous oxide (70% v/v) for 30 min and the tail-flick latency measurement was repeated.

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its antinociceptive action decreases. Therefore, subsequent antinociceptive studies were performed after 30 min of 70% (v/v) nitrous oxide exposure.

Naloxone, the opiate receptor antagonist, blocked nitrous oxide's analgesic effect when systemically administered (fig. 2). Each of the three α_2 adrenergic antagonists (the imidazoline, atipamezole [fig. 3a], the nonimidazoline yohimbine [fig. 3b], and the noncompetitive EEDQ [fig. 3c]), administered systemically, reversed the antinociceptive effects of nitrous oxide. Because the only activity common to atipamezole, yohimbine, and EEDQ is its α_2 adrenoceptor blockade, subsequent regional studies were performed with just one representative of this group. Atipamezole, when administered intrathecally in a dose-dependent manner, blocked the antinociceptive response to nitrous oxide (fig. 4a). Atipamezole, when administered intracerebroventricularly (fig. 4b), did not block the antinociceptive response. Conversely, when administered intrathecally, naxolone had no effect on the antinociceptive response (fig. 5a), whereas when administered intracerebroventricularly it completely blocked the analgesic effect to nitrous oxide (fig. 5b).

Discussion

The main findings of this study indicate that (1) supraspinal, but not spinal, opiate receptors and (2) spi-

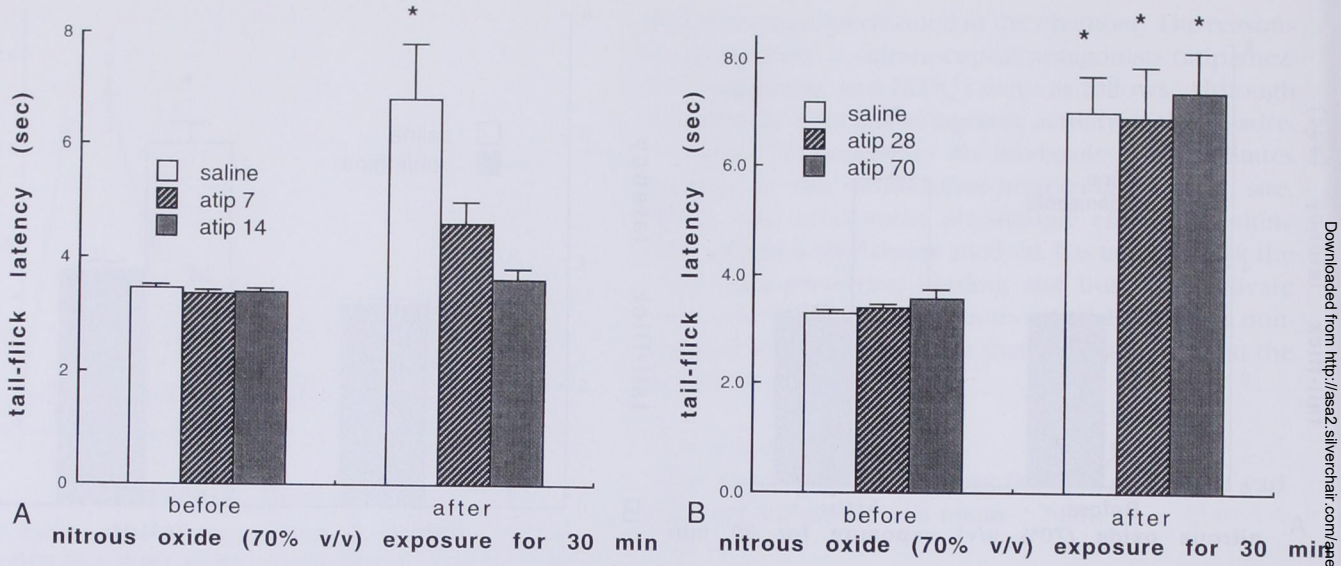

Fig. 4. Effect of local administration of atipamezole on antinociceptive response to nitrous oxide. Six cohorts of rats (n = 6 per group) received cannulae positioned either intrathecally (A) or intracerebroventricularly (B). Rats received injections of either saline or atipamezole (7, or 14 μg in 10 μl intrathecally; 28, 70 μg in 10 μl intracerebroventricularly) 10 min before the latency to eliciting a tail flick in response to a heat stimulus was measured. Subsequently, the animals were exposed to nitrous oxide (70% v/v) for 30 min and the tail-flick latency measurement was repeated.

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nal, but not suprapsinal, α_2 adrenoceptors mediate the antinociceptive action of nitrous oxide in rats. These interpretations are predicated by the fact that localized administration of each antagonist discretely blocks receptors at the site of injection and does not distribute to the other site. This was recently validated for atipamezole, the α_2 adrenoceptor antagonist.[16]

The opiate antagonist, naloxone, was shown to attenuate the analgesic effect of N_2O in mice[1] and subsequently in humans,[2] which led the authors to speculate that opiate receptors mediated the analgesic effect of N_2O. However, the dose used (5.0 $mg \cdot kg^{-1}$ in mice) was much larger than the dose required to block responses to exogenously administered opiate narcotics. This may be important because naloxone itself can exert a hyperalgesic effect at high doses.[17] Using smaller doses of naloxone, other investigators have demonstrated a mild enhancing,[18] a mild attenuating,[19] or no effect[20] on N_2O analgesia in humans. Later studies showed that plasma β endorphin concentrations and [Met^5]enkephalin[21] concentrations in the cerebrospinal fluid increase after N_2O exposure; however, neither the [Met^5]enkephalin[4] nor the endorphin[5,6] findings could be corroborated. Subsequent studies revealed that N_2O increased β-endorphin concentrations along the arcuate propriomelanocortin neuronal system in rats,[22] an effect that was reproduced in an *in vitro* system.[23] This same group demonstrated that nitrous oxide's analgesic effect could be completely attenuated after ablating the periaqueductal gray region in the rat.[24] Subsequently, the ability of naloxone to reverse N_2O analgesia was shown to be stereospecific, suggesting that the drug was acting specifically, presumably at an opiate receptor site.[21] Recently, in a study involving dogs, the levels of two derivatives of the proenkephalin system were found to be elevated in cerebrospinal fluid obtained from the third ventricle in chronically cannulated animals given N_2O.[25] This study differed methodologically from the previous negative studies[4-6] using scientifically rigorous sampling and assay techniques. Therefore, on balance, it seems likely that N_2O does provoke the release of endogenous opiate ligands in the area of the periaqueductal gray.

The mechanism whereby stimulation of opiate receptors in supraspinal regions produce analgesia has been extensively investigated and appears, in part, to involve a descending noradrenergic pathway. Antinociception produced by supraspinally injected morphine is blocked by intrathecally administered antagonists of adrenergic receptors,[7,8] whereas it is enhanced by the

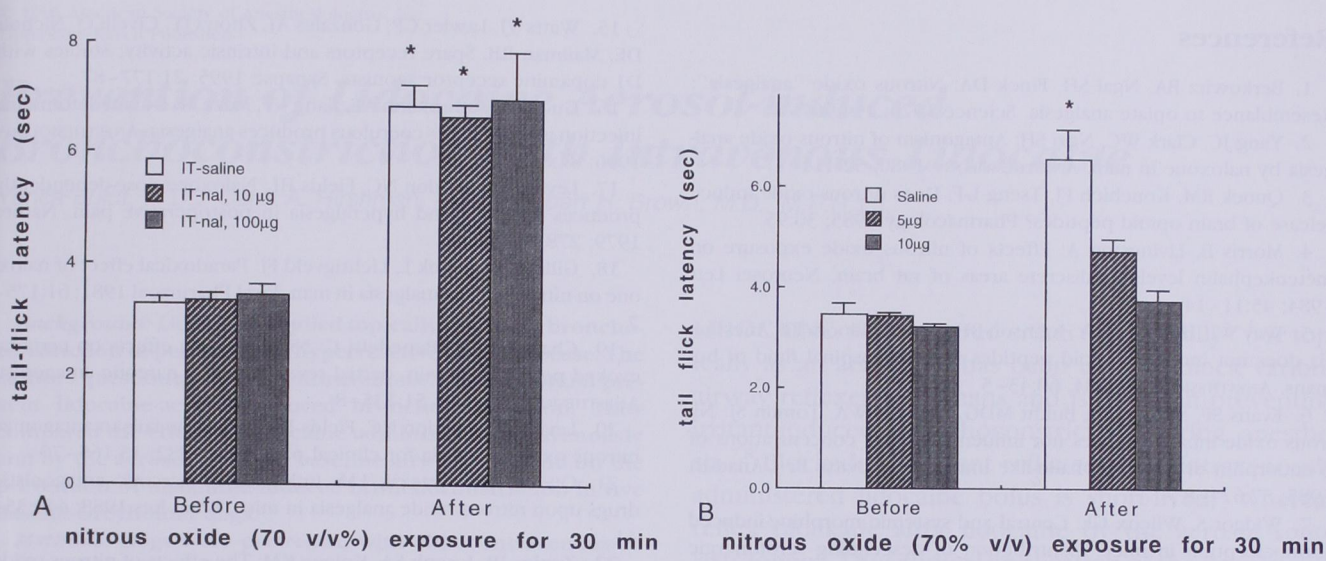

Fig. 5. Effect of local administration of naloxone on antinociceptive response to nitrous oxide. Six cohorts of rats (n = 6 per group) received cannulae positioned either intrathecally (A) or intracerebroventricularly (B). Rats received injections of either saline or naloxone (10, 100 µg, in 10 µl intrathecally; 5, 10 µg in 10 µl intracerebroventricularly) 10 min before the latency to eliciting a tail flick in response to a heat stimulus was measured. Subsequently, the animals were exposed to nitrous oxide (70% v/v) for 30 min and the tail-flick latency measurement was repeated.

Data are expressed as mean ± SEM.

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intrathecal administration of drugs that block the reuptake of norepinephrine.[9] In addition, discrete injection of morphine into the periaqueductal gray increases norepinephrine metabolites in the spinal cord,[10] and its analgesic effects are attenuated by previous depletion of norepinephrine stores in the spinal cord.[11]

A likely descending inhibitory noradrenergic pathway originates in the A7 nucleus in the brainstem. Recently, a functional connection between the opiate receptor-rich periaqueductal gray region and the A7 nucleus was demonstrated.[26] Therefore, based on our findings, we speculate that N_2O provokes the release of endogenous endorphins and enkephalins that stimulate the opiate receptors in the periaqueductal gray region to cause norepinephrine to be released in the spinal cord. This, in turn, activates α_2 adrenoceptors in the dorsal horns to produce an antinociceptive effect.[27]

The duality of involvement of both opioidergic and noradrenergic systems, at unique but different sites, explains the enigmatic "cross-tolerance" findings. Rats and mice develop tolerance to the analgesic effect of N_2O after prolonged exposure to the gas.[28] Biochemically, this is associated with a decrease in opiate-binding sites in the brainstem[29]; despite this, there is no cross-tolerance to the antinociceptive effect of systemically administered morphine. Yet the analgesic response to N_2O is lost in mice made to tolerate morphine. We suggest that in opioid-tolerant animals, the endogenously released endorphins and enkephalins after nitrous oxide exposure fail to activate the desensitized opiate receptors in the periaqueductal gray. Consequently, antinociceptive properties of nitrous oxide are lost. Conversely, although chronic nitrous oxide exposure may desensitize opiate receptors in the periaqueductal gray, spinal opiate receptors will remain unperturbed because at this location the mediating receptor mechanism involves α_2 adrenergic and not opiate receptors. Therefore, in the nitrous oxide–tolerant state, systemically administered opiate narcotics have functional spinal opiate receptors available to transduce the analgesic response even though other possible opiate receptor sites for the analgesic response may have become desensitized.

In conclusion, data from our study suggest that α_2 adrenoceptors in the spinal cord are part of the mediating mechanism for the antinociceptive properties of nitrous oxide. The α_2 adrenoceptors in this spinal site are strategically placed to act as a final common pathway in the antinociceptive response to supraspinal stimulation by endogenous and exogenously applied compounds.[16]

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