Published in final edited form as:

Anesth Analg. 2009 October ; 109(4): 1111–1116. doi:10.1213/ANE.0b013e3181b5a2a7.

Nitrous Oxide-Induced Analgesia Does Not Influence Its Immobilizing Requirements

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Abstract

BACKGROUND—Nitrous oxide (N₂O) acts on supraspinal noradrenergic neurons to produce analgesia, but it is unclear if analgesia contributes to N₂O's immobilizing effects. We tested the hypothesis that N₂O minimum alveolar anesthetic concentration (MAC) is unchanged after selective ablation of supraspinal noradrenergic neurons, or in naïve animals at N₂O exposure timepoints when analgesia is absent.

METHODS—We determined tailflick latency (TFL) and hindpaw withdrawal latency (HPL) under 70% N₂O, N₂O MAC, and isoflurane MAC before and after intracerebroventricular injections of anti-dopamine-β hydroxylase conjugated to saporin (SAP-DBH; *n* = 7), or a control antibody conjugated to saporin (*n* = 5). In a separate group of naïve rats (*n* = 8), N₂O MAC was determined at 25–45 min after initiation of N₂O exposure (during peak analgesia) and again at 120–140 min (after TFL and HPL returned to baseline).

RESULTS—After 30 min of N₂O exposure, TFL and HPL increased significantly but declined back to baseline within 120 min. N₂O did not produce analgesia in rats that received SAP-DBH. However, N₂O and isoflurane MAC were not significantly different between SAP-DBH and control-injected animals (Mean ± SD for N₂O: 1.7 ± 0.1 atm vs 1.7 ± 0.2 atm; isoflurane: 1.6 ± 0.2% vs 1.7 ± 0.2%). In naïve animals, N₂O MAC was not different at the 30 min period compared with the 120 min period (1.8 ± 0.1 atm vs 1.8 ± 0.2 atm).

CONCLUSIONS—Destroying brainstem noradrenergic neurons or prolonged exposure to N₂O removes its analgesic effects, but does not change MAC. The immobilizing mechanism of N₂O is independent from its analgesic effects.

Nitrous oxide (N₂O) is one of the most commonly used anesthetics and analgesics. Its mechanism of analgesic action has been extensively investigated and described.^{1–5} N₂O activates neurons in the periaqueductal gray matter. These neurons project to noradrenergic neurons in the locus coeruleus (LC), which project to dorsal horn neurons in the spinal cord. Activation of the supraspinal noradrenergic neurons results in release of norepinephrine, inhibiting nociceptive transmission in the spinal dorsal horn. When the supraspinal noradrenergic neurons are destroyed, N₂O no longer produces analgesia as determined by tail flick to noxious thermal stimulation.⁴

During continuous exposure to N₂O, analgesia peaks at 30–45 min and then declines such that at 120 min N₂O analgesia is no longer present.⁶ However, N₂O minimum alveolar anesthetic concentration (MAC that produces immobility in 50% subjects) was often determined across exposure times that approached or exceeded 120 min.^{7–9} Therefore, it has not been formally tested whether N₂O's analgesic effects contribute to its immobilizing effects. We hypothesized that the immobilizing potency of N₂O would not change under conditions where N₂O analgesia is absent: after selective destruction of supraspinal noradrenergic neurons, or by comparing MAC values when N₂O analgesia is present with those measured during time periods after analgesia has dissipated.

METHODS

The University of California, Davis animal care and use committee approved this study. Animals were given free access to food and water and maintained on a 12-h light-dark cycle with lights on at 0700.

Intracerebroventricular Injection

Adult male Sprague-Dawley rats (350–450 g) were anesthetized with isoflurane in a chamber and then maintained on isoflurane anesthesia via mask. The head was secured to a stereotaxic frame with ear bars and a mouth piece. Under sterile conditions, a scalp incision was made to expose bregma and a small surrounding area of the skull, in which a small craniotomy was made to permit intracerebroventricular (icv) injections (1.3 mm lateral to midline, –0.8 mm referenced to bregma, and 4.5 mm ventral to the surface of the dura). In a pilot study, we injected three animals with Evans Blue at these coordinates and waited 30 min, and upon necropsy of the brain, dye was found distributed throughout the ventricles in all the three animals.

Saporin conjugated to a monoclonal antibody directed against dopamine-β-hydroxylase (SAP-DBH) was purchased from Advanced Targeting Systems (San Diego, CA) and used to selectively kill noradrenergic neurons.¹⁰ The saporin-DBH complex binds to dopamine-β-hydroxylase on adrenergic terminals and is endocytosed. Saporin then inactivates ribosomes, which results in cell death after 1–2 wk.

Saporin-DBH, or a control immunoglobulin (mouse IgG) conjugated to saporin, was diluted in saline to a concentration of 1 µg/µL. The mixture was placed into a 10-µL microsyringe housing a 27-gauge needle (Hamilton, Reno, NV), which was then secured to a stereotaxic manipulator. We slowly injected 4 µg of saporin-conjugated DBH or control antibody icv over 1–2 min and left the needle tip in place for 10–15 min before retracting the needle and closing the overlying skin with silk sutures. Buprenorphin (0.03 mg/kg) was administered shortly after discontinuing anesthesia. The animals were allowed to recover in their cages for 2 wk before further testing.

Behavioral Nociceptive Testing

Tail flick latency (TFL) and hindpaw withdrawal latency (HPL) were determined as previously described.¹¹ In brief, the animals were placed into acrylic restraining tubes (tailflick) or chambers (hindpaw withdrawal) with an inflow and outflow port for air or N₂O mixed in oxygen, and a third port used for continuous sampling and measurement of N₂O and oxygen concentrations (Ohmeda Rascal II, Helsinki, Finland). Tailflick tubes had an additional hole to permit tail protrusion. Rats were acclimated to the tailflick and hindpaw chambers for 1 h on each of 2 days before the commencement of testing, and for 20 min before each testing period. A custom-built tailflick apparatus was set to produce a baseline TFL of 3–4 s, with an 8 s cutoff time to prevent tissue damage. The thermal hindpaw

withdrawal apparatus (IITC, Woodland Hills, CA) was adjusted to an intensity producing 7–9 s baseline withdrawal latencies when radiant heat was projected onto the mid-portion of the plantar hindpaw (20 s cutoff time). Five trials were made with 2–3 min between trials and averaged for each animal. N₂O (70% with 30% oxygen) was then added to four hindpaw withdrawal chambers or four tail flick tubes at a rate of approximately 7 L/min. During N₂O exposure, TFL or HPL was determined every 30 min for 120 min. The experimenter was blinded with regard to treatment. The rats were then removed and placed into their cages. TFL and HPL were measured in the same animals but on separate days. The order of TFL and HPL testing was reversed for postinjection measurements, and we did not find any significant effects of repeated exposure with testing intervals ≥ 24 h in control animals.

Determination of Minimum Alveolar Concentration

For detailed methodology concerning MAC determination, the reader is referred to Quasha et al.¹² The rats were placed into separate small plastic cages. Two subcutaneous needle electrodes (Grass, West Warwick, RI) were inserted 1 cm apart into the tail and securely taped in place. The cages were placed into a custom-made hyperbaric chamber.¹³ In brief, this consisted of a large thick-walled acrylic cylinder, which had two large aluminum end caps that could be linked by rods and tightened with nuts. The wires of the tail electrodes were attached to electrical pass-throughs in one of the base plates. The wires emerging on the other side were attached to an electrical stimulator. The chamber was filled with 100% oxygen at 1 atm, and then pressurized with N₂O. A small gas sample was removed from the chamber and passed through a calibrated anesthetic agent analyzer (Ohmeda Rascal II). The total N₂O pressure was determined by multiplying the N₂O concentration by the total absolute chamber pressure determined from a pressure gauge attached to the chamber. After a 10–15 min equilibration period, the MAC was determined by passing electrical current through the tail electrodes (70 mA at 100 Hz for 30 s) and observing for movement, which was defined as a pawing motion or movement of the head toward the tail. If the animals moved, then additional N₂O (0.2–0.3 atm) was added to the chamber and the MAC determined again after a 10–15 min equilibration period. If the animals did not move, gas in the chamber was removed via a leak valve (decreasing N₂O partial pressure by 0.2–0.3 atm), and, after equilibration, the noxious stimulus was applied again. N₂O MAC was determined 25–45 min after beginning exposure, corresponding to a time period over which TFL and HPL data were collected when peak analgesia was observed during behavioral testing.

On a separate day, the rats were anesthetized with isoflurane in a chamber and the tracheas intubated. Anesthesia was maintained with isoflurane. End-tidal isoflurane was measured using a calibrated agent analyzer. The isoflurane end-tidal concentration was maintained constant for a 20 min equilibration period, and a 10-in. hemostat applied to the base of the tail and oscillated at approximately 1 Hz for 60 s or until gross and purposeful movement occurred (pawing motion or movement of the head toward the stimulus). An electrical stimulus (70 mA, 100 Hz) was also applied to the tail via subcutaneous needle electrodes (Grass) 2–3 min before or after the tail clamp stimulus, and the order in which we presented the two types of stimuli was randomized. The isoflurane concentration was increased or decreased in 0.2% increments, depending on whether the animal moved, and after a 15–20 min equilibration period, the stimulus was applied again. This process was continued until two isoflurane concentrations were found that just permitted and prevented movement for each stimulus type. The MAC was the average of these values.

A separate group of rats (naïve) was used to assess potential changes in N₂O MAC over time. Using the same electrical tail stimulus as used for the DBH-injected rats, the first N₂O MAC determination (MAC1) was made after 25–45 min of exposure, and the second MAC determination (MAC2) was made after 120–145 min of exposure to N₂O. These timepoints

were chosen because MAC1 corresponded to the time period when we observed peak analgesia during behavioral testing, and MAC2 corresponded to a time period after TFL and HPL had returned to baseline. After the first MAC determination, the animals were left undisturbed until the second MAC determination starting about 120 min later.

Immunohistochemistry

Animals were deeply anesthetized with isoflurane, perfused through the aorta with phosphate buffered saline followed by 4% paraformaldehyde, and the brains were removed. The brains were placed into paraformaldehyde for 24–48 h and then 30% sucrose. The brains were cut in 50- μ m sections and every third section collected in a multi-well container for immunohistochemical staining of tyrosine hydroxylase (TH) following the method described by Colombari et al.¹⁴ Briefly, sections were treated in 0.5% hydrogen peroxide in 0.02 M phosphate buffered saline for 15 min, followed by incubation in 3.5% normal horse serum (Vector Laboratories, Burlingame, CA) for 30 min. They were then incubated for 24 h with a monoclonal TH antibody (1:2000, ImmunoStar, Hudson, WI) in 1.5% normal horse serum and 0.2% Triton X-100, followed by incubation in biotinylated horse antimouse IgG for 1 h (1:200 with 0.1% Triton X-100; Vector Labs). The tissue was then subjected to an avidin-biotin-complex procedure (Elite Vectastain, Vector Labs) and immunoreactivity was visualized by a diaminobenzidine stain that resulted in a golden-brown cytoplasmic TH reaction product. Stained sections were mounted on glass microscope slides, dehydrated, cleared, and coverslipped. An investigator blinded as to treatment examined the sections under the light microscope (Nikon Eclipse E400) equipped with a digital camera (Micropublisher 5.0). Photomicrographs were taken of the LC and A5/A7 cell groups. In control animals, TH immunoreactivity was very dense in the LC, making it difficult to count individual TH-stained cells, whereas TH staining was essentially absent in the LC in the DBH-saporin treated animals. For this reason, we made no attempt to quantify cell numbers or density of immunoreactivity.

Statistical Analysis

Data are presented in figures as mean and standard error of the mean. Within group, TFL and HPL under N₂O were compared with baseline and across time of exposure using an analysis of variance with timepoint set as a factor and animal designated as a random-effects factor, followed by Tukey multicomparisons. A preinjection versus postinjection comparison for TFL and HPL was made in the SAP-DBH group at the 30 min timepoint using a two-tailed paired *t*-test. Postinjection comparisons between SAP-DBH and control groups were made at the 30 min timepoint for TFL, HPL, and MAC using an unpaired, two-tailed *t*-test. A two-tailed, paired *t*-test was used to compare MAC values at the two different timepoints in the naïve group. A *P* value <0.05 was considered significant.

RESULTS

Before receiving icv injections, TFL and HPL significantly increased in the control group (*n* = 5) and the SAP-DBH group (*n* = 7) after 30 min exposure to 70% N₂O (Fig. 1, solid symbols; *P* < 0.001 in all cases). However, under continued N₂O exposure, latencies decreased back toward baseline so that by 90 min TFL and HPL were no longer significantly different from baseline, but significantly decreased from latencies at 30 min (when peak analgesia occurred). Two weeks after icv injection, control rats continued to display the same profile of analgesia over time during N₂O exposure. In contrast, the group that received the SAP-DBH did not demonstrate significant analgesia at any timepoint as indicated by both TFL and HPL measurements (Fig. 1, open symbols).

After icv injections, the N₂O MAC was not different between the SAP-DBH ($n = 7$) and control-injected ($n = 5$) groups (Mean $\pm$ SD: 1.7 ± 0.1 atm vs 1.7 ± 0.2 atm, respectively). Isoflurane MAC also was not different between these two groups, and isoflurane MAC for using electrical tail stimulation was not different from isoflurane MAC using noxious mechanical stimulation. Electrical tail stimulation isoflurane MAC was $1.6\% \pm 0.2\%$ for the SAP-DBH group and $1.7\% \pm 0.2\%$ for the control group. Tail clamp isoflurane MAC was $1.6\% \pm 0.2\%$ for the SAP-DBH group and $1.7\% \pm 0.2\%$ for the control group.

Histological analysis confirmed that TH staining in the LC was nearly absent in all SAP-DBH injected animals, demonstrating ablation of brainstem noradrenergic neurons. However, control-injected animals exhibited dense LC TH staining. Figures 2A–C show photomicrographs of transverse brainstem sections with TH-immunolabeled noradrenergic LC neurons in three randomly selected rats receiving control icv injections. Figures 2D–F show examples of similar brainstem sections from three randomly selected rats treated with SAP-DBH, displaying an absence or paucity of TH-immunolabeling.

In the naïve animal group ($n = 8$), N₂O MAC1 was not different from MAC2 determined at the later timepoint (MAC1: 1.8 ± 0.2 atm vs MAC2: 1.8 ± 0.2 atm).

DISCUSSION

The main findings of this study were that loss of noradrenergic neurons in the brainstem, or prolonged N₂O exposure, removed the analgesic properties of N₂O, confirming prior studies,^{4,6} while having no effect on its immobilizing properties. Based on indirect clinical evidence, it has been theorized that the analgesic effects of N₂O are separate from its immobilizing effects.¹⁵ Our present data provide direct evidence in support of this hypothesis. We discuss these findings related to the proposed mechanism of action of N₂O as well as the site of action of anesthetic-induced immobility.

N₂O is proposed to produce analgesia by a supraspinal action that has been comprehensively described in review articles.^{3,15} This N₂O-evoked descending inhibition of nociceptive transmission is thought to be engaged initially by the release of corticotropin-releasing factor (CRF) from the hypothalamus.¹⁶ Because antagonism of *N*-methyl-D-aspartate-type glutamate receptors elevates CRF levels,¹⁷ this effect is presumably due to *N*-methyl-D-aspartate receptor inhibition, one of N₂O's most potent effects on ligand-gated ion channels.¹⁸ Although ketamine also potently inhibits nicotinic acetylcholine receptors,^{19–21} acetylcholine typically increases CRF and antagonism of cholinergic receptors suppresses its release.^{22,23} N₂O-evoked CRF secretion is proposed to cause the release of endogenous opioids that disinhibit midbrain periaqueductal gray excitatory output neurons, which excite LC bulbospinal adrenergic neurons to inhibit nociceptive transmission at the level of the spinal cord,^{1,4,5,24} through activation of GABAergic dorsal horn neurons.²⁵

Our data show that the loss of LC noradrenergic neurons in the brainstem (and possibly partial reductions in other adrenergic nuclei, such as A5 and A7) removed the analgesic effects of N₂O, but the immobilizing properties of N₂O remained unchanged. These findings suggest that N₂O produces immobility via a different mechanism from that which produces analgesia. Many anesthetics produce immobility by action in the spinal cord. We have found that neurons in the ventral horn (where locomotor interneurons and motoneurons reside) are more depressed by N₂O, isoflurane, halothane, and propofol than are neurons in the dorsal horn.^{7,26,27} Still, we cannot exclude the possibility that N₂O acts on some other descending pathway that contributes to immobility.

We additionally investigated whether selective destruction of supraspinal noradrenergic neurons altered isoflurane requirements. Because the use of a hyperbaric chamber for N₂O

MAC testing necessitated the use of a noxious electrical stimulus, we compared isoflurane MAC in these same animals using both a typical noxious mechanical stimulus and the electrical stimulus. MAC was the same for both stimulus types, as previously reported.²⁸ We cannot exclude the possibility, however, that N₂O MAC might have been different between the groups that had used a mechanical or thermal stimulus.

Another reason for testing isoflurane MAC in DBH and control-injected animals was that Kingery et al.²⁹ found that isoflurane produced supraspinal pronociceptive and antinociceptive effects that are mediated in part by noradrenergic neurons. Our data suggest, however, that these actions also do not impact the immobilizing potency of isoflurane, consistent with a spinal action. Again, this direct spinal action likely occurs in ventral motor circuits and is not an “antinociceptive” effect in the dorsal horn.^{26,27,30} However, isoflurane’s pronociceptive effects have also been shown to be mediated through inhibition of nicotinic acetylcholine receptors,³¹ which were presumably not impacted by the adrenergic lesion.

The analgesia produced by N₂O peaks within 15–45 min but wanes after that period such that analgesia is absent by approximately 120 min after the beginning of exposure. In our prior studies determining N₂O MAC,¹³ we often did not finish our MAC determinations until well after 60 min (unpublished data). Thus, we wondered how the diminished analgesic effect impacted MAC. We presently found that MAC determined during the period when the peak of N₂O-induced analgesia is present was not different from MAC determined at a time when analgesia should be absent. These data further suggest that N₂O produces immobility by a pathway and mechanism separate from that by which it produces analgesia.

In summary, we found that removal of N₂O-induced analgesia by destruction of noradrenergic neurons in the brainstem, or by time-dependent tachyphylaxis, had no effect on the immobilizing properties of N₂O. We conclude that N₂O produces immobility independent from its analgesic effects.

Acknowledgments

The authors are indebted to Mirela Iodi-Carstens for her expertise and skill in immunohistochemistry.

Supported by National Institute of Health grants R01 GM 078167 (to SLJ), R01 DE 13685 (to EC), P01 GM 047818 and R01 GM 061283 (to JFA).

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Figure 1.

Line graphs showing mean effects of nitrous oxide on tail flick latency (A and B) and hindpaw withdrawal latency (C and D) to noxious thermal stimuli over time. Graphs are shown for animals receiving control injections (A and C) or saporin-anti-dopamine- β -hydroxylase (SAP-DBH) injections (B and D). Before receiving intracerebroventricular (icv) injections (solid symbols), animals displayed significant analgesia in tailflick and hindpaw withdrawal tests after 30 min of nitrous oxide exposure, which returned to baseline over time in the presence of continued nitrous oxide exposure. Two weeks after icv injection (open symbols), control animals continued to display analgesia at the 30 min timepoint, however animals undergoing ablation of adrenergic neurons with SAP-DBH did not display analgesia at any timepoint under nitrous oxide. *Significantly different from baseline latencies ($P < 0.005$ in all cases). +Significantly different from 30 min preinjection within-group values ($P < 0.015$) and significantly different from postinjection control animals at 30 min timepoint ($P < 0.001$).

Figure 2.

Photomicrographs showing tyrosine hydroxylase immunostaining from three control animals injected intracerebroventricular (icv) with a control antibody conjugated to saporin (A–C) and three animals injected icv with saporin-anti-dopamine- β -hydroxylase (D–F). Magnified views (A2–F2) are shown to the right of the sections taken from each respective animal.