Advances in Understanding the Actions of Nitrous Oxide

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Nitrous oxide (N₂O) has been used for well over 150 years in clinical dentistry for its analgesic and anxiolytic properties. This small and simple inorganic chemical molecule has indisputable effects of analgesia, anxiolysis, and anesthesia that are of great clinical interest. Recent studies have helped to clarify the analgesic mechanisms of N₂O, but the mechanisms involved in its anxiolytic and anesthetic actions remain less clear. Findings to date indicate that the analgesic effect of N₂O is opioid in nature, and, like morphine, may involve a myriad of neuromodulators in the spinal cord. The anxiolytic effect of N₂O, on the other hand, resembles that of benzodiazepines and may be initiated at selected subunits of the γ-aminobutyric acid type A (GABA_A) receptor. Similarly, the anesthetic effect of N₂O may involve actions at GABA_A receptors and possibly at N-methyl-D-aspartate receptors as well. This article reviews the latest information on the proposed modes of action for these clinical effects of N₂O.

Key Words: Nitrous oxide; Pharmacology; Anesthesia; Analgesia; Anxiolysis.
For nearly 150 years since its discovery, N₂O has been used by clinicians without a clear knowledge of its mechanisms of action. Only over the last 20–30 years has there been any significant research elucidating the mechanisms of the analgesic, antianxiety, and anesthetic effects of N₂O. In 1985, Eger published the first comprehensive book on N₂O, gathering all available information up to that time and providing an excellent summary of clinical and research issues. Since then, a large amount of research has been added, clarifying a number of issues about the actions of N₂O. A Medline search for research manuscripts on the pharmacology of N₂O revealed 2439 articles between 1940 and 1980; a similar search conducted recently uncovered 5801 articles between 1981 and 2006.

This timely review will update readers on the current state of knowledge of the pharmacokinetic and pharmacodynamic aspects of N₂O. The relevant clinical uses of N₂O as an analgesic, anxiolytic, and anesthetic drug will be explored.

ANALGESIA

Subanesthetic concentrations of N₂O produce only analgesic and anxiolytic effects without unconsciousness. Analgesic N₂O has a long history of use in obstetrics for relief of labor pain. N₂O is also used for self-administered analgesia in cancer patients, preemptive analgesia, and to alleviate pain and discomfort associated with a number of medical procedures, including intra-articular drug injection, peripheral intravenous cannulation, sigmoidoscopy, colonoscopy, ophthalmologic procedures, and biopsy procedures. In Europe, Entonox (BOC Group), a 50% N₂O: 50% oxygen mixture, is widely used in emergency medical care of patients in accident scenes and during ambulance transportation.

The mechanism of the analgesic effect of N₂O is gradually being clarified through elucidation of the antinociceptive effect of N₂O in animals. Because animals are unable to report a reduction in sensation of pain, analgesia is more properly determined in animals as antinociception or a diminished responsiveness to a noxious stimulus.

The Opioid Hypothesis of N₂O Antinociceptive Action

It was as early as 1943 when the analgesic effect of N₂O was judged to be comparable to that of opioid analgesic drugs—30% of N₂O was deemed to be equipotent as 10–15 mg of morphine. It was not until the mid-1970s that it was first reported that N₂O-induced antinociception in mice and rats was sensitive to blockade by the narcotic antagonist naloxone. N₂O-induced analgesia in human subjects was also antagonized by naloxone.

A large number of studies have established an important role for opioid receptors in the periaqueductal gray (PAG) area of the midbrain in pain modulation. The N₂O-induced analgesic effect could be completely ablated after lesioning the PAG in the rat or following microinjection of opioid antagonists into the PAG. Although these results clearly implicate opioid receptors in mediation of N₂O-induced antinociception and analgesia, it is appreciated today that opioid receptors are not a monolithic species. There are multiple opioid receptors that are capable of mediating pain relief, and the specific subtypes of opioid receptors that mediate the antinociceptive effects of N₂O appear to depend on various factors including the species and/or strain, the regions of the brain, and the experimental noxious stimulus. In the mouse abdominal constriction test, N₂O antinociception was unaffected by either µ or δ opioid antagonists but was sensitive to blockade by drugs with antagonist properties at κ opioid receptors. A κ opioid ligand also protected N₂O antinociception in mice from antagonism by an irreversible, nonselective opioid antagonist. The κ opioid receptor subtype appears to be involved at both the supraspinal and spinal cord levels in suppression of chemical noxious stimulation, which is supported by observations that N₂O-induced antinociception was antagonized by supraspinal and spinal pretreatment with antisera against the endogenous κ opioid ligand, dynorphin (DYN).

In the rat hot plate test, µ and ε opioid receptor subtypes appear to perform a main function at the supraspinal level, as demonstrated by the effectiveness of µ and ε opioid antagonists to reduce N₂O-induced antinociception. The opioid connection was further strengthened by reports of morphine-tolerant animals being cross-tolerant to N₂O. The fact that cross-tolerance was unilateral in that N₂O-tolerant animals were not cross-tolerant to morphine led Berkowitz et al to hypothesize that N₂O might work through stimulating the neuronal release of endogenous opioid peptides. Chronic treatment with morphine results in desensitization of opioid receptors and/or signal transduction mechanisms, hence resulting in cross-tolerance to N₂O, which relies on the same opioid receptors. Chronic treatment with N₂O results in a tolerance that is attributable to excessive depletion of endogenous opioid peptide stores, such that a subsequent exposure to N₂O is unable to release sufficient quantities of opioid peptides to cause antinociception. The chronic exposures to N₂O in these tolerance investigations were not sufficient for inducing the same changes at the receptor and/or signal levels that were observed in the chronic morphine studies.
The first chemical evidence for \( N_2O \)-induced release of opioid peptides did not emerge until nearly 10 years following the first report of naloxone antagonism of \( N_2O \)-induced antinociception. Exposure to 75% \( N_2O \) for 60 minutes increased by 2-fold the amount of immunoreactive methionine-enkephalin (ME) in fractions of perfusate collected from ventricular-cisternally perfused rats.\(^{37} \) At the end of \( N_2O \) exposure, the levels of immunoreactive ME returned to baseline. This led to the conclusion that \( N_2O \) was capable of inducing the neuronal release of either ME itself or a ME-like peptide in the rat brain. Later studies also showed that \( N_2O \) increased \( \beta \)-endorphin concentrations in the arcuate pro-priomelanocortin neuronal system in rats,\(^{38} \) a result that was reproduced in an in vitro system.\(^{39} \)

The most extensive studies have been conducted in the mouse abdominal constriction test. The opioid peptide released by \( N_2O \) was identified as DYN in experiments utilizing rabbit antisera against rat opioid peptides. \( N_2O \) antinociception was antagonized by intracerebroventricular pretreatment with antisera against DYN\(_{1-8}\) and DYN\(_{1-13}\), but not ME or \( \beta \)-endorphin.\(^{34} \) In a subsequent study, it was discovered that \( N_2O \) antinociception was also sensitive to antagonism by intrathecal pretreatment with antisera to DYN\(_{1-8}\), DYN\(_{1-13}\), and ME.\(^{35} \) These findings are consistent with studies reporting that activation of supraspinal \( \kappa \) opioid receptors causes a release of ME in the spinal cord.\(^{40} \) Therefore, in this one experimental model, it appears that \( N_2O \) evokes its antinociceptive effect through the supraspinal release of various DYNs, which are the endogenous ligands of \( \kappa \) opioid receptors, and spinal release of DYNs and ME.

Involvement of Nitric Oxide in \( N_2O \) Antinociception

Nitric oxide (NO) is a naturally occurring gas that only recently has been recognized as an endogenous biological regulator of great significance. Science magazine declared NO as “The Molecule of the Year” for 1992.\(^{41} \) There is evidence that NO released from nitrergic neurons seems to regulate the release of a variety of transmitters (acetylcholine, catecholamines, excitatory and inhibitory amino acids, serotonin, histamine, and adenosine) in the brain.\(^{42} \) \( N_2O \) antinociception was antagonized in dose-related fashion by a series of L-arginine analogs that competitively inhibit NO synthase (NOS), including L-N\(^{G} \)-nitro arginine. This antagonism was stereoselectively reversed by administration of L-arginine but not D-arginine. L-N\(^{G} \)-nitro arginine had no such interaction with morphine or the \( \kappa \) opioid agonist U-50,488H.\(^{43} \) Later studies demonstrated that \( N_2O \)-induced antinociception was more specifically antagonized by pretreatment with a selective inhibitor of neuronal NOS\(^{44} \) or an antisense oligodeoxynucleotide directed against neuronal NOS.\(^{45} \) Nitric oxide also appears to play a key role in opioid peptide release. A tangential study was designed to test this hypothesis. If \( N_2O \) antinociception in the abdominal constriction model is caused by stimulated release of DYNs, which then activate \( \kappa \) opioid receptors, and if NO appears not to influence \( \kappa \) opioid receptor or signal transduction, then it is possible that NO influences the stimulated release of DYNs.\(^{46} \) (See Figure 1)

Further evidence of the importance of NO in \( N_2O \) antinociception emerged from experiments in inbred mouse strains. \( N_2O \) evoked a concentration-dependent antinociception in various mouse strains, including the C57BL/6 inbred strain, but DBA/2 inbred mice exposed to identical levels of \( N_2O \) responded with only a weak antinociceptive effect.\(^{47,48} \) When mice were exposed to \( N_2O \), there was increased whole-brain NOS activity—as quantified by radioconversion of [\(^{14} \)C]L-arginine to [\(^{14} \)C]L-citrulline—in the C57BL/6 mouse but not the DBA/2 mouse.\(^{49} \) This apparent correlation between antinociceptive responsiveness and increase in NOS enzyme activity was recently confirmed in mice selectively bred for low sensitivity to \( N_2O \)-induced antinociception.\(^{50} \)

Quantitative trait loci analysis in C57BL/6 and DBA/2 mice, their B6D2F\(_{1}\) offspring, 22 BXD recombinant inbred strains (derived from the progenitors), and 600 offspring bred from the F\(_2\) generation identified 2 markers from chromosomes 2 and 5 that were significantly correlated with \( N_2O \) antinociception and 1 marker from chromosome 18 that was suggestive.\(^{48,51} \) It is significant that the genetic control of neuronal NOS is localized to mouse chromosome 5 in the same vicinity as one of the significant markers in the quantitative trait loci analysis.\(^{52} \) Determination of other NOS-related factors may also be located in the same area of the chromosome.

Therefore, it seems likely that in the mouse abdominal constriction model, NO provokes the release of endogenous opiates (DYN peptides) playing a mediatory role in the antinociceptive effect of \( N_2O \). The location of the nerve terminals from which DYN is released and the location of the \( \kappa \) opioid receptors have not been determined.

Descending Pathways Activated by \( N_2O \)

Fujinaga and Maze\(^{53} \) hypothesized that the release of endogenous opioid peptides and the subsequent stimulation of opioid receptors activate descending pathways that modulate nociceptive processing in the spinal cord. There are several steps to this process, as demonstrated by a series of elegant studies conducted in rats. A nor-
N$_2$O-induced analgesia. N$_2$O is thought to stimulate the neuronal release of endogenous opioid peptide or dynorphins (DYNs); the molecular aspects of how N$_2$O initiates this process are as yet unknown. The presynaptic nerve terminal takes up L-arginine (L-Arg), which is converted by the enzyme nitric oxide synthase (NOS) to L-citrulline (L-Cit) and nitric oxide (NO). NO appears to be involved in the stimulated release of DYNs. DYNs traverse the synaptic cleft and activate postsynaptic opioid receptors, which belong to the 7-transmembrane–spanning, G protein–coupled superfamily of receptors.

The disinhibited noradrenergic pathway appears to modulate spinal nociceptive processing by 2 divergent pathways (Figure 2). One population of $\alpha_2$ adrenergic receptors is located on second-order neurons in the pain pathway, whereas the other is located on inhibitory GABA-ergic interneurons in the spinal cord. This dual involvement of GABA as pronociceptive supraspinally and antinociceptive spinally is consistent with experimental findings. N$_2$O-induced antinociception was antagonized by intracerebroventricular administration of muscimol, a GABA type A (GABA$_A$) agonist, and intrathecal administration of gabazine, a GABA$_A$ antagonist.

Immunohistochemical and in situ hybridization identification of the immediate early gene c-fos or the FOS protein that it encodes can be used to map functional activation in discrete brain regions of rats following physiological, pharmacological, or psychological stimulation. N$_2$O exposure increases c-Fos expression in the pontine noradrenergic nuclei as well as the spinal cord. N$_2$O-induced c-Fos expression in the spinal cord was colocalized to cells containing the rate-limiting enzyme in the synthesis of GABA. Expression of c-Fos in these regions was antagonized by opioid receptor blockade and also by stimulation of GABA$_A$ receptors in the PAG. Microinjection of opioid antagonist and GABA$_A$ agonist into the pontine A7 nuclei also inhibited N$_2$O-induced expression of c-Fos in the spinal cord as well as attenuate N$_2$O-induced antinociception.

**Tolerance to N$_2$O Antinociception**

As with many centrally mediated drug effects, continuous administration of N$_2$O results in development of tolerance to the antinociceptive effect of N$_2$O in experimental animals and to the analgesic effect of N$_2$O in human subjects. Studies in different rat strains have provided valuable insight into the development of tolerance to N$_2$O. The Fischer rat strain exhibits a robust antinociceptive response to N$_2$O but does not show acute tolerance, whereas the Lewis rat strain is poorly responsive to N$_2$O-induced antinociception. In addi-
tion to differential sensitivity to N₂O, the Fisher and Lewis rats also differ in neurochemistry and behavioral reactions to other centrally active drugs.⁵⁷⁶⁸ Compared to the Fisher rat, the poorly responsive Lewis strain has lower basal levels of endogenous opioid peptides and does not respond with an increase in opioid peptide levels following the administration of morphine.⁶⁸ This is also consistent with findings that maintenance of high levels of opioid peptide by inhibiting enkephalinase enzyme can prevent the development of acute tolerance to N₂O in rats.⁶⁹

ANXIOLYSIS

In dentistry, subanesthetic concentrations of N₂O are routinely used to produce moderate sedation for dental surgery in anxious patients.⁷⁰ Minimal and moderate sedation (or conscious sedation, as was the previous terminology used) is mediated by the administration of agents causing alterations in the level of consciousness, cognition, motor coordination, degree of anxiety, and physiological parameters. It is not defined by specific medications or their doses but instead by the patient’s response: the patient must retain the ability to respond purposefully to verbal commands either alone or accompanied by light tactile stimulation.⁷¹

In pediatric dentistry, N₂O is an invaluable tool in managing the mildly to moderately anxious child. The ease of its administration, its wide margin of safety, its analgesic and anxiolytic effects, and, most of all, its rapid reversibility make it an ideal drug for use in children.⁷²–⁷⁵ The most recent survey of the active members of the American Academy of Pediatric Dentistry by Houpt⁷⁶ reported that 61% of 1758 respondents used N₂O/O₂ with other sedative agents. The American Academy of Pediatric Dentistry recognizes nitrous oxide/oxygen inhalation as a safe and effective technique to reduce anxiety, produce analgesia, and enhance effective communication between a patient and health care provider.⁷¹

There is evidence that the relaxation and relief from anxiety during inhalation of N₂O is a specific anxiolytic effect that is independent of the analgesic action of N₂O. The mechanisms involved are not yet completely understood.

The Benzodiazepine/GABA Receptor Hypothesis of N₂O Anxiolysis

N₂O evokes patterns of behavioral response that are reminiscent of the effects of benzodiazepines in different animal models of experimental anxiety, including the mouse staircase test,⁷⁷–⁷⁹ the mouse elevated plus maze,⁸⁰ the mouse light/dark exploration test,⁸¹ the mouse hole board,⁸² the rat social interaction test,⁸³ and the rat conditioned defensive burying test.⁸⁴ N₂O- and benzodiazepine-induced anxiolytic-like behaviors were equally sensitive to antagonism by the benzodiazepine binding site blocker flumazenil.⁷⁹,⁸³,⁸⁴ Mice that are rendered tolerant to benzodiazepines by daily treatment with escalating doses of chloridiazepoxide are cross-tolerant to the anxiolytic-like behavioral response to N₂O.⁷⁹,⁸⁰ These findings strongly implicate that the anxiolytic effect of N₂O is associated with brain benzodiazepine mechanisms.

**Figure 2.** Influence of N₂O on descending inhibitory pathways. N₂O induces release of endogenous opioid peptides (EOP) that activate opioid receptors on γ-aminobutyric acid (GABA)-ergic pontine nuclei. This pathway, in turn, activates descending noradrenergic system in the dorsal horn of the spinal cord that directly inhibits or indirectly inhibits (through a GABA interneuron) nociceptive processing at the level of the primary afferent and second-order neurons that transmit sensory signals up the ascending nociceptive pathway.
Signaling Pathway That Mediates Anxiolytic-like Activity

Because benzodiazepines work through facilitation of GABA-ergic inhibitory neurotransmission, research was conducted to determine involvement of GABA_A receptors in N_2O anxiolysis. In the light/dark exploration test, N_2O- and chloralhydrate-induced increases in time spent in the light compartment as well as the number of transitions were blocked by the benzodiazepine antagonist flumazenil and the GABA_A antagonist SR-95531 (2-[3-carboxypropyl]-3-amino-6- [4-methoxyphenyl]-pyridazinium bromide). Consistent with the known interaction between benzodiazepine and GABA_A receptors, these findings indicate that GABA_A receptors mediate the anxiolytic-like effects caused by chloralhydrate and N_2O activation of benzodiazepine receptors. This is also supported by observations that N_2O-induced depression of visual evoked potentials is antagonized by a benzodiazepine inverse agonist.43

N_2O- and benzodiazepine-induced anxiolytic-like effects in animal models of anxiety are also sensitive to antagonism by inhibition of NOS, a family of enzymes responsible for the synthesis of NO. Studies in the elevated plus maze revealed that the increased open-arm activity produced by N_2O and chloralhydrate was blocked by a nonselective NOS inhibitor; this antagonism was stereoselectively reversed by L-arginine.46,47 In the light/dark exploration test, selective neuronal NOS inhibitors antagonized both N_2O- and chloralhydrate-induced increases in the time spent in the light compartment.43,81 The antagonism of N_2O was duplicated by NO scavenger hemoglobin89 as well as an antisense oligodeoxynucleotide against neuronal NOS.89 These findings suggest that NO plays a key role in the anxiolytic signaling mechanism downstream from the benzodiazepine/GABA_A receptor complex.88 The key role of NO in anxiolysis was also evidenced by the anxiolytic-like effects of a centrally administered NO donor.80

The soluble 3’,5’-cyclic guanosine monophosphate (cGMP)-dependent pathway has been identified by many studies as the main signal transduction pathway of NO.42 In experiments in the light/dark exploration test, N_2O anxiolysis was blocked by the guanylyl cyclase inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one) and the GMP-dependent protein kinase (PKA)/cGMP-dependent protein kinase (PKG) inhibitor H8 (N-[2-(methyl-aminophenyl)-5-isouquinoline sulfonamide HCl) and was potentiated by the cyclic GMP phosphodiesterase inhibitor zaprinast.91 cGMP is known to act upon several different targets: cGMP-dependent protein kinases (PKG), cGMP-faded cation channels, or cGMP-regulated phosphodiesterase.92 N_2O-induced anxiolytic-like behavior was significantly attenuated by inhibitors of PKG but not cAMP-dependent protein kinase (PKA).93 (See Figure 3)

Although there is evidence that stimulation of GABA_A receptors activates an anxiolytic signaling pathway that includes an enzyme sequence of NOS, soluble guanylyl cyclase, and PKG, how N_2O acts at the molecular level to stimulate the BZ binding site and GABA_A receptor is not yet known. In a manner similar to how N_2O activates opioid receptors, it is plausible that N_2O may induce neuronal release of endogenous benzodiazepine factors that then stimulate the GABA_A receptor.

ANESTHESIA

N_2O has a well-known role in medical history because it was the first drug used for surgical anesthesia. Despite its limited anesthetic potency, N_2O is the most widely used general anesthetic agent. With a minimum alveolar concentration of 104% in humans, N_2O by itself would require high volume percentage and hyperbaric conditions to achieve anesthesia in 50% of subjects.94 Therefore, because of its low potency, in clinical practice, N_2O is generally used to reduce the minimum alveolar concentration of a second inhalation agent for anesthesia and increase the rate of induction (ie, the second gas effect95) and to provide or augment the analgesic component of general anesthesia.

General anesthetics like N_2O have long been hypothesized to act in a nonspecific manner on neuronal membranes, alter membrane fluidity, and/or influence ion channels. But more recently, it has been suggested that general anesthetics might act on one or more superfamilies of ligand-gated ion channels that include GABA_A, glycine, nicotinic acetylcholine, 5-hydroxytryptamine, and glutamate receptors.96,97 Among the ligand-gated ion channels, the GABA_A receptor is considered to be a prime target of volatile and intravenous anesthetics. Several anesthetics are known to potentiate the activity of GABA at inhibitory GABA_A receptor. N_2O itself has been reported to affect various ligand-gated ion channels.98–100

N-methyl-D-aspartate (NMDA)-type glutamate receptors have recently emerged as a possible target of inhalation anesthetic drugs. Studies in cultured rat hippocampal neurons revealed that N_2O inhibited NMDA-activated currents in a dose-dependent manner but had no effect on GABA-activated currents.101 Consistent with NMDA antagonism, N_2O is reported to up-regulate binding of NMDA radioligand in the cerebral cortex.102 N_2O also inhibited excitotoxic neurodegeneration that is mediated through NMDA receptors. Similar to other NMDA antagonists, the neurotoxic effect of N_2O is age-dependent and is sensitive to attenuation by GABA-erg-
Figure 3. Mechanism of N₂O-induced anxiolysis. N₂O is thought to cause activation of the benzodiazepine (BZ) binding site as its effects are blocked by flumazenil. This action facilitates γ-aminobutyric acid (GABA) activation of its binding site, resulting in chloride ion influx. The increased chloride ion concentration in the neuron might cause activation of calmodulin (CaM), which then activates the enzyme nitric oxide synthase (NOS). NOS converts the amino acid L-arginine (L-Arg) to L-citrulline (L-Cit) and NO, which stimulates the enzyme soluble guanylyl cyclase producing the second messenger cyclic guanosine monophosphate (cyclic GMP). The cyclic GMP, in turn, stimulates a cyclic GMP-dependent protein kinase (PKG) that leads to the anxiolytic drug effect.

SUMMARY

It is apparent from the above discussion that N₂O has multiple mechanisms of action that underlie its varied pharmacological properties. Current research indicates that the analgesic effect of N₂O appears to be initiated by stimulated neuronal release of endogenous opioid peptides, with subsequent activation of opioid receptors and descending GABA and noradrenergic pathways that modulate nociceptive processing at the spinal level. The anxiolytic effect of N₂O involves activation of the GABAA receptor through the benzodiazepine binding site, although whether N₂O acts directly or indirectly upon the latter targets remains uncertain. The anxiolytic pathway that is stimulated includes a segment that involves a sequence of 3 key enzymes, NOS, soluble guanylyl cyclase, and PKG. The anesthetic effect of N₂O appears to be caused by inhibition of NMDA glutamate receptors and removing its excitatory influence in the nervous system.

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